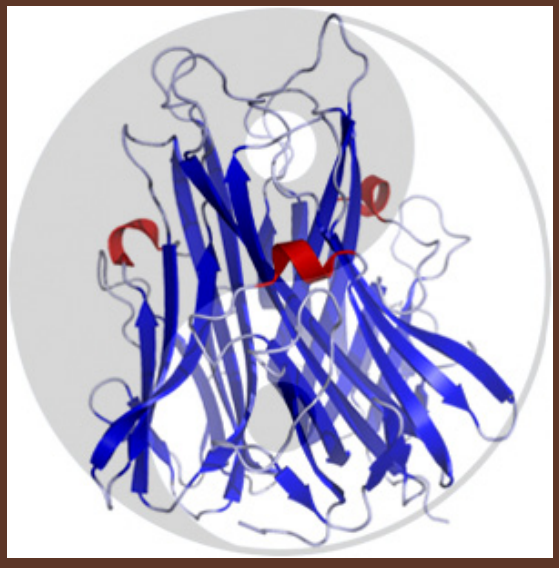
Functional implications of TNF-receptor pathways on regulatory T cells



Xin Chen

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The research presented in this thesis was performed at the Laboratory of Molecular Immunoregulation, National Cancer Institute, NIH, Frederick, USA (contract HHSN261200800001E) and the Department of Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

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by

Xin Chen
Born on February 14, 1962
In Huangmei, China

Supervisor:

Prof. dr. M.G. Netea

Prof. Dr. J.J. Oppenheim (US National Cancer Institute, Frederick, USA)

Co-supervisor:

Dr. L.A.B. Joosten

Doctoral Thesis Committee:

Prof. dr. R.P. Pickkers

Prof. dr. I. Joosten

Prof. dr. P.W.M. Hermans

Functional implications of TNF-receptor pathways on regulatory T cells

Proefschrift

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aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
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om 14.30 uur precies

door

Xin Chen
geboren op 14 februari 1962
te Huangmei, China

achterzijde

Promotoren: Prof. dr. M.G. Netea

Prof. dr. J.J. Oppenheim (US National Cancer Institute, Frederick, USA)

Copromotor: Dr. L.A.B. Joosten

Manuscriptcommissie:

Prof. dr. R.P. Pickkers

Prof. dr. I. Joosten

Prof. dr. P.W.M. Hermans

To my wife Lu Yang, son Siqi Chen and Mom

Contents

Chapter 1

Introduction

Contrasting immunopathogenic and immunoprotective effects of TNF

The pleiotropic cytokine tumor necrosis factor-alpha (TNF) is a major participant in the initiation and orchestration of complex events in inflammation and immunity (1). The effects of TNF are mediated by two structurally related, but functionally distinct receptors, TNFR1 (or p55) and TNFR2 (or p75) (2). In contrast to the ubiquitous expression of TNFR1, TNFR2 is more restricted to lymphocytes (3). TNFR1 with its death domain (DD) is the primary signaling receptor on most cell types and accounts for the majority of the proinflammatory, cytotoxic and apoptotic effects classically attributed to TNF (4, 5). In contrast, TNFR2 lacks an intracellular death domain and predominantly mediates signals promoting lymphocyte activation and proliferation (6, 7).

TNF has well-documented proinflammatory effects. Nevertheless, increasing evidence reveals that TNF also has unexpected anti-inflammatory and immunosuppressive effect, especially after prolonged exposure [reviewed in ref.(8-10)]. Anti-TNF therapy is clearly beneficial for most of rheumatoid arthritis (RA) patients. However, perhaps reflecting the strikingly contrasting activities of TNF, anti-TNF therapy in patients with RA and inflammatory bowel disease (IBD) is accompanied by the development of lupus and neuroinflammatory diseases in a minority of patients (11). Furthermore, multiple sclerosis (MS) patients treated with anti-TNF agents resulted almost uniformly in immune activation and exacerbation of disease (11). To date, the cellular and molecular mechanisms underlying the contrasting proinflammatory and immunosuppressive effect of TNF, as well as anti-TNF agents, in autoimmunity remain to be clarified and this is the main focus of the research described in this thesis.

Immune equilibrium between regulatory T cells and effector T cells determines the outcome of autoimmune responses

Extensive studies provide compelling evidence that $CD4^{+}FoxP3^{+}$ regulatory T cells (Tregs) play an indispensable role in maintaining immune homeostasis and in suppressing deleterious excessive immune responses (12). The induction of Treg suppressive activity is antigen specific and requires stimulation through the TCR, however, the suppressive function of Tregs is not antigen specific (13). Therefore, a wide range of immune responses can be inhibited by Tregs through “bystander” suppression (14). It is known that the cellular targets of Tregs include CD4 cells (15), CD8 cells (16), NK cells (17), NKT cells

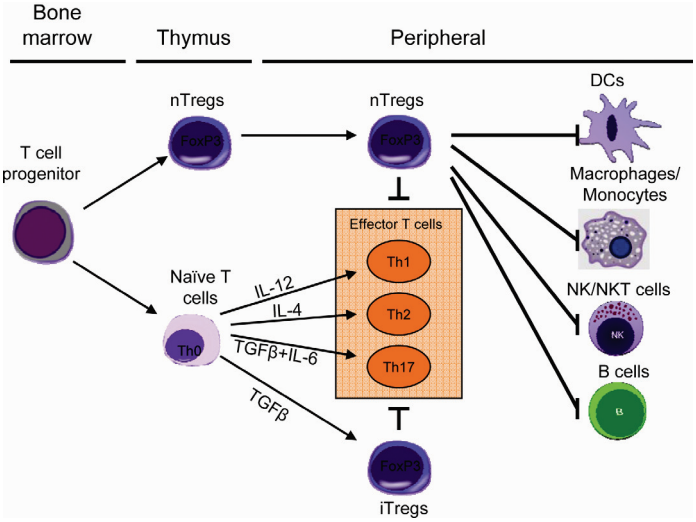

Figure 1: Generation and cellular targets of Tregs. Naturally occurring Treg cells (nTregs) are phenotypically and functionally matured in the thymus from lymphoid progenitor cells. In peripheral, naive CD4 cells can be converted into FoxP3-expressing Tregs (induced Tregs, or iTregs) by TGFβ. Both nTregs and iTregs are able to inhibit the activation of effector T lymphocytes (Teff cells), including both CD4 and CD8 T cells. Furthermore, Treg cells also potently inhibit immunological function of DCs, monocytes/macrophages, NK cells and NK T cells, as well as B cells.

(18), DCs (19), macrophages (20) and B cells (21). The *in vitro* suppressive activity of Tregs depends on cell-to-cell contact (15), and several molecules, such as IL-10, TGFβ, CTLA-4, IDO and granzyme/perforin are reported to contribute to the suppressive activity of Tregs [reviewed in ref. (22), Figure 1]. Activation of Tregs has been reported in various types of inflammatory responses, which may represent a negative feedback mechanism to curtail excessive inflammation and prevent self-tissue destruction (23-26). However, the molecular basis of Treg-activating effect of inflammation remains elusive. I hypothesized that TNF interaction with its receptors had an important biological effect on the balance between T-effector and T-regulatory cells. Therefore, I have asked several important questions with regard to the potential effects of TNF/TNFR pathways on the biological function of Tregs and its consequential effect in the modulation of inflammation:

i. Can TNF, through the interaction with its receptors, promote proliferation and function of Treg cells?
ii. Which TNF receptor pathway is responsible for this effect?

iii. What is the phenotypic and functional implications of expression of TNF receptors on the surface of Treg cells, as well as on T-effector cells?
iv. Does TNF play a role in the stabilization of immunosuppressive phenotypes of Tregs in the inflammatory environment?

To answer the first question, I have initiated a study shown in **Chapter 2** in which the effect of TNF on the proliferation and immunosuppressive function of Tregs was examined. Since TNFR2 is preferentially expressed by Tregs, I consequently characterized the phenotypic and functional implications of surface TNFR2 expression on mouse Tregs, as shown in **Chapter 3**, as well as on human Tregs, as shown in **Chapter 4**. Providing the important role of TNFR2 in the activation of Tregs, in **Chapter 5** I further studied the effect of TNF on the expression of TNFR2, as well as other co-stimulatory TNFRSF molecules, on Tregs. In order to assess the role of TNF-TNFR2 pathway on the stability of Treg phenotype in the inflammatory environment, a colitis model induced by transfer of naive CD4 cells into lymphopenic mouse was studied, as presented in **Chapter 6**. In **Chapter 7**, in order to evaluate the overall role of TNF-TNFR2 pathway on the activation of whole CD4 population which contains both Teffs and Tregs, we studied the functional consequence of TNFR2 expression on Teffs in the presence of Tregs.

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Chapter 2

Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse $CD4^+CD25^+$ regulatory T cells

Xin Chen, Monika Bäumel, Daniela N. Männel, O. M. Zack Howard, and Joost J. Oppenheim

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Abstract

Although TNF is a major proinflammatory cytokine, increasing evidence indicates that TNF also has immunosuppressive feedback effects. We have demonstrated in this study that, in both resting and activated states, mouse peripheral $CD4^+CD25^+$ T regulatory cells (Tregs) expressed remarkably higher surface levels of TNFR2 than $CD4^+CD25^-$ T effector cells (Teffs). In co-cultures of Tregs and $CD4^+CD25^-$ T effector cells (Teffs), inhibition of proliferation of Teffs by Tregs was initially transiently abrogated by exposure to TNF, but longer exposure to TNF restored suppressive effects. Cytokine production by Teffs remained continually suppressed by Tregs. The profound anergy of Tregs in response to TCR stimulation was overcome by TNF, which expanded the Treg population. Furthermore, in synergy with IL-2, TNF expanded Tregs even more markedly up-regulated expression of CD25, FoxP3 and phosphorylation of Stat5, and enhanced the suppressive activity of Tregs. Unlike TNF, IL-1beta and IL-6 did not upregulate FoxP3 expressing Tregs. Furthermore, the number of Tregs increased in wild type, but not in $TNFR2^{-/-}$ mice following sublethal cecal ligation and puncture (CLP). Depletion of Tregs significantly decreased post-CLP mortality. Thus, the stimulatory effect of TNF on Tregs resembles the reported co-stimulatory effects of TNF on Teffs, but is even more pronounced because of the higher expression of TNFR2 by Tregs. Moreover, our study suggests that the slower response of Tregs than Teffs to TNF results in delayed immunosuppressive feedback effects.

Introduction

TNF is a pleiotropic cytokine that is a major participant in the initiation and orchestration of inflammation and immunity (1). TNF has been shown to have proinflammatory effects, nevertheless, increasing evidence reveals TNF to also have unexpected immunosuppressive effects. For example, TNF has been considered as a major proinflammatory mediator of sepsis (1). This cytokine is markedly elevated in human infectious diseases (2) and is reported to be required for survival following cecal ligation and puncture (CLP) (3), which represents a mouse model of polymicrobial peritonitis simulating human sepsis (4). However, both TNF and TNFR-deficient mice fail to develop the usual sepsis-induced immunosuppression after CLP (5). In addition in chronic inflammatory states TNF-deficient mice also exhibit more severe inflammation (6-8). We have therefore tested the hypothesis that the suppressive effects of TNF on the inflammatory response may involve Tregs.

Tregs actively restrain the inflammatory response, suppress development of autoimmune diseases and dampen a wide spectrum of immune responses (9, 10). Tregs express TNFR2 (11) suggesting that TNF may either overcome or promote the activity of Tregs directly. Indeed, it has been reported that neutralization of TNF restored Treg function and reduced the spontaneous apoptosis of Tregs in rheumatoid arthritis (RA) patients (12, 13). In neonatal NOD mice, administration of TNF also promoted the development of diabetes accompanied with reduced number and impaired function of Tregs (14). Paradoxically, injection of young adult NOD mice with TNF suppressed the development of diabetes and expanded Tregs (14). In one report TNF failed to have any effect on *in vitro* co-cultures of human Tregs and Teffs (12), while in another study TNF blocked inhibition of Teffs by Tregs (15). These studies suggest TNF may play an important role in either the down- or up-regulation of Treg activity, but the precise effect of TNF on Tregs remains elusive.

Activation of TNFR2 has direct costimulatory effects on TCR-mediated mouse T cell responses and consequently reduces the activation threshold of antigen-driven CD4 T cell responses (16). We found initially that the signal mediated by TNFR2 effectively stimulated Teffs when co-cultured with Tregs, which may enable TNF-stimulated Teffs to escape from Treg-mediated inhibition and consequently mount a TNF-dependent inflammatory response. Indeed, it was recently reported that TNF also overcomes the immunosuppressive effect of mesenchymal stem cells (MSCs) on T cell proliferation (17). Furthermore, anti-TNF is a well known therapeutic for a number of inflammatory autoimmune conditions. Thus, liberating Teffs from Treg-mediated inhibition may account

for proinflammatory effects of TNF. However, there are paradoxical reports that prolonged exposure to TNF actually is protective in some autoimmune states (7, 8, 18) suggesting that duration and dose of TNF may modify its effects on Teffs and Tregs. Therefore we investigated the effects of different times and doses of TNF exposure on co-cultures of Tregs and Teffs, and the response of Tregs in TNFR2-deficient mice to a septic challenge to determine if and when TNF stimulated Tregs to reassert their suppressive capacity.

Materials and Methods

Mice and reagents. Female C57BL/6 (CD45.2) and C57BL/6 Ly5.2 congenic mice (CD45.1) and BALB/c mice, 8 to 12 wk old, were provided by Animal Production Area of the NCI (Frederick, MD). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.). Mice deficient in TNFR2 (19) were kept and bred in the animal facility of the University of Regensburg. Antibodies purchased from BD PharMingen (San Diego, CA) consisted of FITC-anti-CD4 (GK1.5), PE-anti-CD25 (PC61), PE-anti-Phospho-Stat5 , purified anti-CD3 (145-2C11), purified anti-CD16/CD32 (2.4G2) and anti-CD28 (35.71). PE anti-mouse/rat Foxp3 Staining Set (FJK-16s) was purchased from eBioscience. PE-anti-TNFR1 and PE-anti-TNFR2 were obtained from Serotec Ltd (Oxford, UK). Purified anti-CD25 (PC61) was a generous gift of W. Falk (University of Regensburg). Recombinant mouse cytokines (IL-2, TNF, IL-1β, and IL-6) were purchased from PeproTech (Rocky Hill, NJ).

Purification of cells. $CD4^+$ cells were purified with Mouse CD4 (L3T4) microbeads and LS column (Miltenyi Biotec Inc., Auburn, CA). $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were purified from lymph node (inguinal, axillary and mesenteric regions) cells using Cytomation MoFlo cytometer (Fort Collins, CO), yielding a purity of ~98% for both subsets. T-depleted spleen cells were used as APCs and were prepared by depletion of $CD90^+$ cells with anti-mouse CD90 MicroBead and LD column (Miltenyi Biotec Inc.). APC were irradiated with 3000 R.

Flow Cytometry. After treatment with anti-CD16/CD32 Ab, cells were incubated with appropriately diluted antibodies. For CFSE labelling assay, cells were labelled with 2 μM CFSE for 8 min at room temperature. FACS analysis was performed on a FACScan (BD Biosciences, Mountain View, CA) using CellQuest software.

In vitro cell culture and proliferation assay. $CD4^+CD25^-$ T cells (5×10^4 cells/well) were seeded in a U-bottom 96-well plate in the medium with 2×10^5 cells/well of APCs plus 0.5 μg/ml of soluble anti CD3 Ab. $CD4^+CD25^+$ T cells were added to the wells at desired ratio to $CD4^+CD25^-$ cells. Cells were pulsed with 1 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) per well for the last 6 h of culture period. In some settings, cells were stimulated with 2 μg/ml of soluble anti-CD3 antibody (without APCs). In a parallel experiment, the supernatants were collected and cytokine measurement was performed by analysis of supernatants with SearchLight Mouse Cytokine Array (Pierce Biotechnology, Woburn, MA). In some experiments, IL-2 (10 ng/ml) was supplemented to the medium, with or without TNF (10 ng/ml). In some experiments, $CD4^+CD25^-$ T cells from C57BL/6 Ly5.2 (CD45.1) mice were labelled with CFSE and then were co-cultured with $CD4^+CD25^+$ T cells from C57BL/6 (CD45.2) mice. The co-cultures were stimulated with APC from C57BL/6 (CD45.2) mice and 0.5 μg/ml of soluble anti CD3 Ab. The proliferation of $CD4^+CD25^-$ T cells was assessed by CFSE dilution using flow cytometry, gating on $CD45.1^+$ cells. In some experiments, CFSE-labelled Tregs or CFSE-labelled Teffs were cultured alone or CFSE-labelled Teffs were co-cultured with unlabelled Tregs, or vice versa unlabelled Teffs were co-cultured with CFSE-labelled Tregs at a "physiological" ratio (ratio of Teff/Treg was 10:1) with IL-2 or IL-2 plus TNF. After incubation for 72 h, the proliferation of CFSE-labelled cells was analyzed by flow cytometry. In some experiments, purified Tregs were cultured with medium alone or with TNF (10 ng/ml) for 16 h. The resultant intracellular expression of FoxP3 was analyzed by FACS. In some settings, purified Tregs and Teffs were cultured with IL-2 (10 ng/ml) or IL-2 plus TNF (10 ng/ml, each) for 24 h to 72 h, after washing, the intracellular expression of FoxP3 was analyzed by FACS, and the immunosuppressive activity of cultured Tregs was determined by co-culturing with freshly isolated Teffs, either using the [^{3}H] thymidine incorporation assay or CFSE-labelling assay.

Cecal ligation and puncture (CLP). C57BL/6 mice were anesthetized by i.p. injection of 75 mg/kg Ketanest® (Parke, Davis & Company, Munich, Germany) and 16 mg/kg Rompun® (Bayer AG, Leverkusen, Germany). The cecum was exteriorized and the distal end (< 30%) was ligated and punctured once with a needle (0.4 mm diameter) to achieve a sublethal CLP as described previously (2). For Treg depletion mice received 200 μg anti-CD25 (PC61) or rat IgG, respectively, in 500 μl PBS 3 days prior to CLP. Splenocytes were isolated on day 1 or 2 after CLP.

Statistical analysis. Comparisons of data were analyzed by two-tailed Student's *t* test using Graphpad Prism 4.0 or using the Mann-Whitney-U-Test. The log-rank test was used to

compare Kaplan-Meier survival curves.

Results

Expression of TNF receptors on resting and activated Tregs and Teffs

TNF mediates its biological functions through its receptors: TNFR1 and TNFR2; the latter is largely confined to cells of the immune system (20). Unlike TNFR1 which contains a death domain in its cytoplasmic tail, the primary function of TNFR2 is to promote cellular proliferation and survival (1). We were able to confirm the data of Kim and Teh (21) showing that mouse splenic $CD4^+$ as well as $CD8^+$ T cell subpopulations express moderately high levels of TNFR2. The expression of TNFR2 was further increased by T cell activation (data not shown). $CD4^+$ splenocytes from TNFR2-deficient mice were less

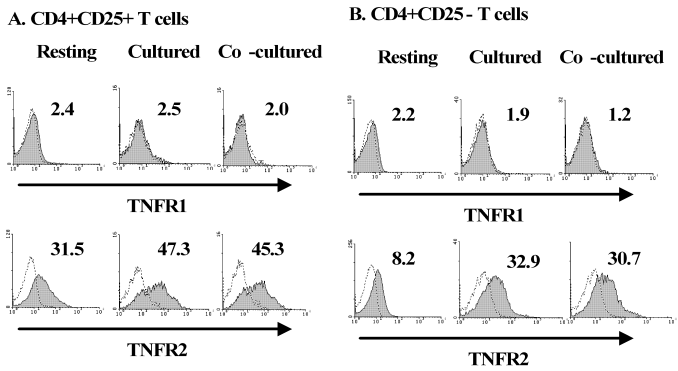

Figure 1. **Surface expression of TNFRs on Teffs and Tregs**. $CD45.1^+$ Tregs (5×10^4 cells/well, A), $CD45.2^+$ Teffs (5×10^4 cells/well, B) were cultured alone (cultured) or co-cultured together (co-cultured, ratio of Teff/Treg was 2:1). The cells were stimulated with APC and anti-CD3. After 48 h, the surface expression of TNFR1 and TNFR2 was analyzed by gating on $CD4^+$ and $CD45.1^+$ (for Tregs) or $CD4^+$ and $CD45.2^+$ (for Teffs) population. Freshly isolated cells (resting) were used for comparison. The number in the histogram is the percentage of TNFR positive cells. Data shown are representative of 2 separate experiments.

able than those from wild type mice to proliferate in response to anti-CD3 and anti-CD28 (data not shown). It has been reported that human thymic Tregs expressed high levels of surface TNFR2 (11). Consistent with this report, we found considerably higher constitutive expression of surface TNFR2 on mouse peripheral Tregs (32%) than on Teffs (8%) (Fig.1). Upon TCR stimulation, expression of this receptor on Tregs was further increased to 47% and to 33% on activated Teffs. Furthermore, the activation-dependent elevation of TNFR2 expression by Teffs was not reduced by the presence of Tregs at a ratio of Teffs to Tregs of 2:1. Unlike TNFR2, the surface expression of TNFR1 was barely detectable by FACS on

either resting or activated Teffs or Tregs (Fig.1).

Effect of TNF on co-cultures of Teffs and Tregs 8 h) exposure of TNF on the outcome of *in vitro* co-cultures of Trergs and Teffs. Exogenous

Next we evaluated the effect of relative short term (4 TNF markedly increased cell proliferation in the co-cultues (Fig. 2A). This effect of TNF was observed over various ratios of Tregs to Teffs (data not shown). To exclude potential interference by APC, co-cultures of Teffs and Tregs were stimulated with soluble anti-CD3 alone. TNF also increased the proliferation by these APC-free co-cultures (Fig. 2B). Thus, the proliferative effect of TNF is due to direct action on CD4 subsets. TNF not only enhanced proliferation of Teffs, but it also overcame the non-responsiveness of Tregs to TCR stimulation (Fig. 2C), presumably by co-stimulatory signalling through TNFR2 which reduces the threshold of both Teffs and Tregs to TCR-dependent activation. At 48 h, TNF stimulated proliferation of Teffs and reduced the inhibition of Teffs by Tregs by [^{3}H] thymidine incorporation assay in a dose dependent manner (Fig. 2D~E). In contrast, more prolonged exposure of co-cultures to TNF (72 h) restored the inhibition of proliferation of Teffs by Tregs (Fig. 2D~E).

Since both Teffs and Tregs incorporated thymidine by 48 h and 72 h of incubation in the presence of TNF, we further examined the impact of TNF on the proliferation of co-cultured congenic CD45.1$^+$ Teffs by CFSE dilution assay. As shown in Fig 2F, CFSE-dilution assay were more delayed compared to the [3H]thymidine incorporation assay, since DNA synthesis occurs prior to cell replication. The CFSE assay showed considerable proliferation by 72 h of incubation because 90% of Teffs by this time replicated. This was reduced to 22.2% by the addition of Tregs. When Teffs alone were cultured with TNF, the proportion of replicating Teffs similarly was 93.9%, but the number of cells resulting from multiple replications by CFSE assay was greater than in the absence of TNF. In the presence of Tregs and TNF, replicating Teffs decreased to 38.9%. Based on the proportion of replicating Teffs, the percent inhibition by Tregs at 48 h was 57.6% and this was reduced to 37.3% in the presence of TNF. At 72 hours, the percent inhibition by Tregs was 75.3% and 58.6%, in the absence and presence of TNF, respectively. The reduction in inhibition by TNF was therefore 35.2% and 20.8% at 48 h and 72 h, respectively. Since TNF stimulated multiple rounds of division by Teffs at 72 h, the precise percent inhibition by Tregs in the presence of TNF at this time point was actually greater than 58.6%, and consequently, the reduction in inhibition by TNF was actually less than 20.8%. Similar

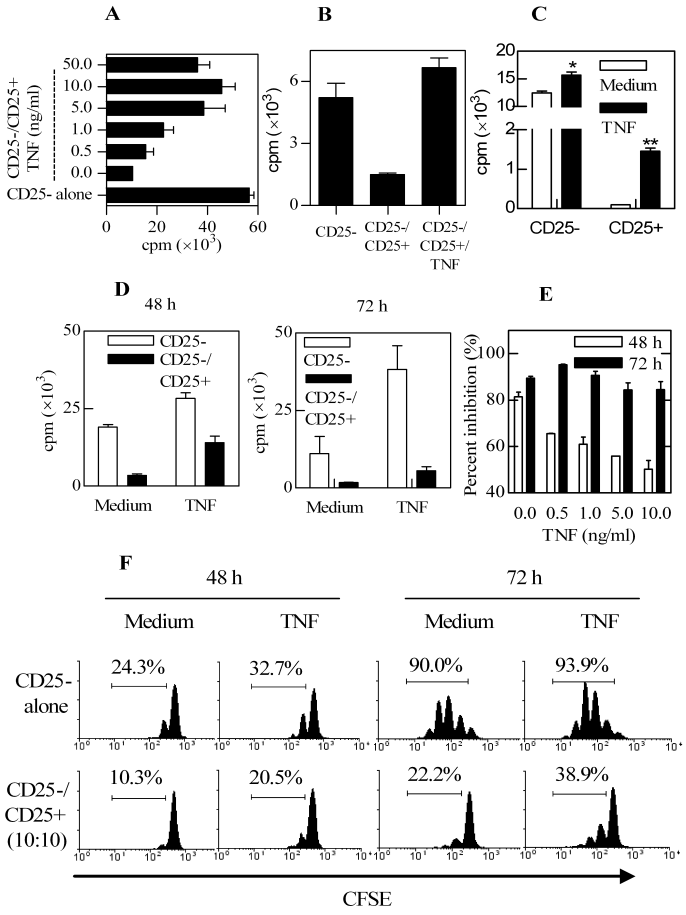

Figure 2. **Effect of exogenous TNF on the lymphocyte proliferation in co-cultures**. (A) Teffs (5×10^4 cells/well) cultured alone or co-cultured with Tregs (ratio of Teff/Treg was 2:1), were stimulated with APC and anti-CD3, in the presence of increasing levels of TNF. (B) co-cultures of Teffs (5×10^4 cells/well) and Tregs (2.5×10^4 cells/well) were stimulated with soluble anti-CD3 alone, in the presence or absence of 10 ng/ml TNF. (C) 5×10^4 cells/well of Teffs or Tregs were cultured alone and stimulated with APC/anti-CD3, with or without 10 ng/ml of TNF. The proliferative response was determined after 48 h culture (A~C). (D) Cells were cultured and stimulated as in (A) for 48 h or 72 h, in the presence or absence of 10 ng/ml of TNF. (E) Cells were cultured and stimulated as in (A) for 48 h or 72 h in the presence of increasing concentration of TNF. The data shown are percent inhibition (%). (F) FACS-sorted Teffs from C57BL/6 Ly5.2 (CD45.1) mice were labelled with CFSE and were cultured alone or co-cultured with Tregs from C57BL/6 (CD45.2) mice, in the presence or absence of 10 ng/ml of TNF. The cells were stimulated with $CD45.2^+$ APC and anti-CD3. After incubation for 48 h and 72 h, proliferation by Teffs was analyzed by gating on CD45.1+ cells. The data shown are representative of at least 3 separate experiments with similar results.

results obtained in three separate experiments. Altogether these results lead us to conclude that the shorter duration of treatment with TNF (48 h) showed partial abrogation of inhibition of Teffs by Tregs, while more prolonged exposure to TNF (72 h) restored Treg-mediated inhibition.

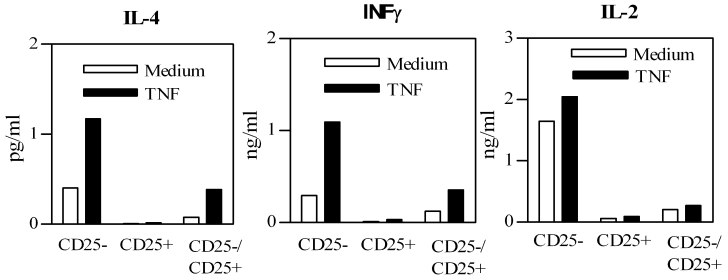

Figure 3: Effect of exogenous TNF on cytokine production by co-cultures. 5×10^4 cells/well of Teffs and Tregs were cultured alone or co-cultured at a ratio of 1:1. The cells were stimulated with APC and anti-CD3, in the presence or absence of 10 ng/ml of TNF. After incubation for 48 h, IL-4, IFNγ, and IL-2 in the supernatant were determined by multiplex mouse cytokine measurement. The data shown are representative of at least 3 separate experiments with similar results.

The Treg-mediated suppression of cytokine production by Teffs was detected early on. At 48 h of incubation, in the absence of TNF, Tregs produced very low cytokine levels in response to TCR stimulation and markedly inhibited the capacity of Teffs to produce IL-4, INFγ, and IL-2 by 81%, 67%, and 87% (Fig. 3). Addition of TNF led to a 3-fold increase in IL-4 and IFNγ production by purified Teffs and partially restored the production of IL-4 and IFNγ (but not IL-2) in co-cultures. However, in the presence of Tregs plus TNF, the percent inhibition of IL-4, INFγ, and IL-2 production by Teffs was just still as high as 77%, 67%, and 86%, respectively, which is comparable to the inhibitory effect of Tregs in the absence of TNF (Fig. 3). Therefore, short term exposure to TNF for up to 48 h reversed inhibition of Teffs proliferation by Tregs, but capacity of Tregs to inhibit cytokine production by Teffs occurred even at the earlier time point.

TNF synergizes with IL-2 in activating Tregs

In order to determine whether TNF acted directly on Tregs, TNF was added to the purified Tregs cultured without APC and anti-CD3. Both FoxP3 expression and immunosuppressive activity of unstimulated Tregs rapidly decreased after 16 h incubation in medium, but addition of TNF partially prevented this decrease in FoxP3 expression (Fig. 4A) and partially maintained the immunosuppressive function of Tregs (data not shown). However, we could not evaluate the effect of longer incubation periods under such condition since TNF was not able to support prolonged survival of Tregs.

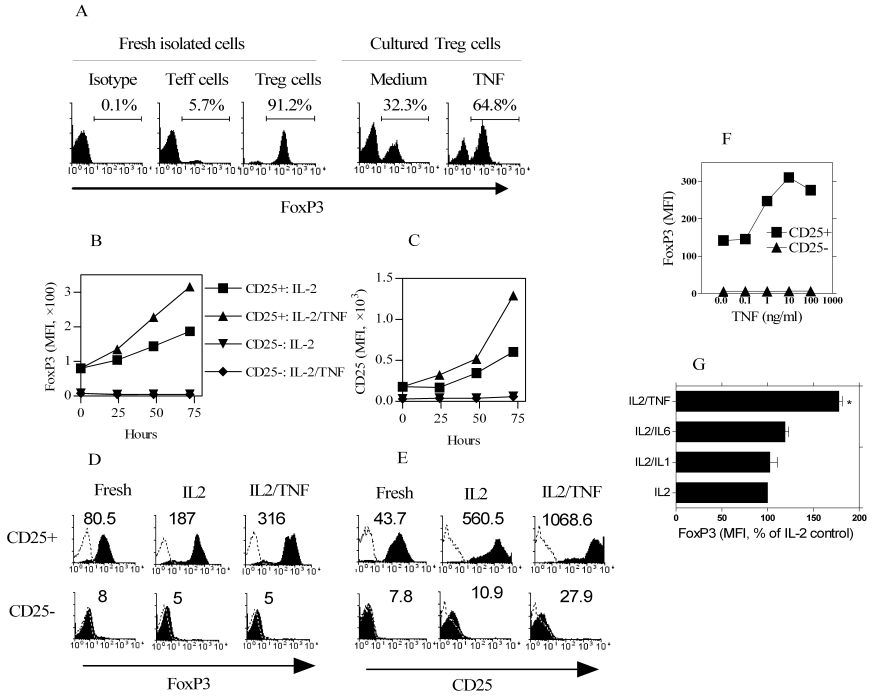

Figure 4. TNF, synergizing with IL-2, selectively activates Tregs. (A) Tregs were cultured with RPMI-10 alone (medium) or 10 ng/ml of TNF (TNF). 16 h later, cells were harvested and expression of FoxP3 was analyzed by FACS, compared with freshly isolated cells. (B-E) Tregs or Teffs were cultured in the medium supplemented with 10 ng/ml IL-2, with or without 10 ng/ml TNF for 24 h, 48 h, and 72 h. Kinetic expression of FoxP3 (B) and CD25 (C) by Tregs and Teffs cultured in IL-2, with or without TNF, was determined by FACS. Typical histogram of FoxP3 (D) and CD25 (E) expression by 72 h cultured Treg or Teffs, compared with freshly isolated cells. Dashed line shows isotype control, solid histogram shows FoxP3 (D) or CD25 (E) Ab staining. (F) Dose-response of TNF (0.1 to 100 ng/ml) on FoxP3 expression by Tregs and Teffs cultured with medium containing IL-2 (10 ng/ml) for 72 h. The number in the histogram is MFI (mean fluorescence intensity). The data are representatives of at least 3 separate experiments with similar results. (G) Effect of proinflammatory cytokines on FoxP3 expression by IL-2 cultured Tregs. 10 ng/ml of IL-1β, or IL-6, or TNF on FoxP3 expression (MFI) by Tregs cultured with IL-2 for 72 h. The data are shown as percentage of IL-2 culture alone (%), summarized from 3 separate experiments (Mean±SEM). Compared with IL-2 culture alone, *P<0.05.

It has been amply demonstrated that IL-2 is crucial for survival of *in vitro* cultured Tregs (22). We therefore used IL-2 to enable us to observe a more prolonged *in vitro* effect of TNF on Tregs. As predicted, IL-2 alone increased the expression of FoxP3 and CD25 by Tregs with time. This effect of IL-2 was markedly enhanced by the addition of TNF, in a

time-dependent (Fig. 4B~E) and dose-dependent manner (Fig. 4F). The up-regulation of FoxP3 by TNF was unique, since other inflammatory cytokines such as IL-1β and IL-6 did not share this property (Fig. 4G). These data indicated that TNF, in combination with IL-2, markedly increased Tregs FoxP3 and CD25 expression. In contrast, Teffs did not show changes in FoxP3 or CD25 expression in response to IL-2/TNF (Fig. 4B~D), suggesting TNF can not support de novo differentiation of Tregs from the non-Treg population.

We further determined the effect of TNF on the expansion of the physiological level of ~10% Tregs present in purified CD4 cells. After 72 h of culture with IL-2 alone, the proportion of FoxP3$^+$ cells increased by 2-fold above the level in freshly isolated CD4 cells. The expression level of FoxP3 (MFI) was also dramatically enhanced by TNF (Fig. 5A).Addition of IL-2 plus TNF increased the proportion of FoxP3-expressing cells by 4-fold. The proportion of FoxP3$^+$ cells did not increase further after 5 to 7 days culture, but remained high at a 30 to 40% level (data not shown). CFSE-labelling assays indicated that IL-2 induced the proliferation of a small population (~ 10%) of Tregs. Supplementation of IL-2 with TNF further increased the proportion of proliferating cells by 4-fold over IL-2 alone in cultures of Tregs and in co-cultures of Teffs and Tregs at a 10:1 ratio (Fig. 5B). In

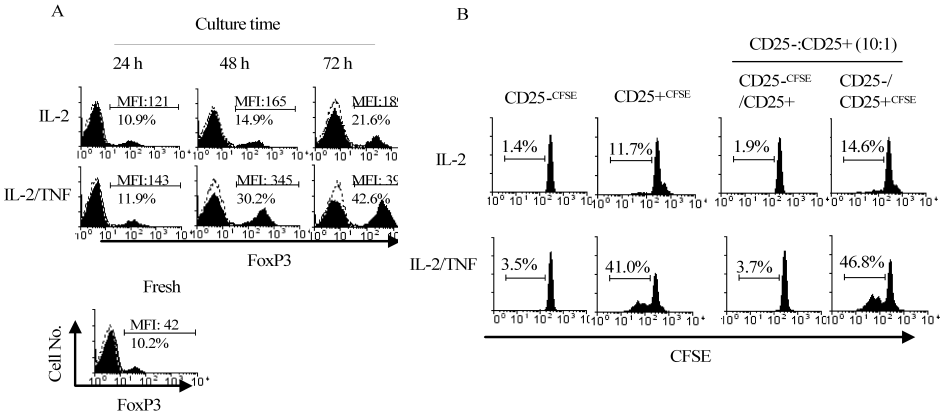

Figure 5. In combination with IL-2, TNF preferentially expands Tregs *in vitro*. (A) Purified CD4 cells (pooled from LNs and spleen) were cultured with 10 ng/ml IL-2, with or without 10 ng/ml TNF for 24 h, 48 h, or 72 h. The expression of FoxP3 was analyzed by FACS. Mean fluorescence intensity (MFI) and the percentage of positive cells are indicated in the histograms. (B) CFSE-labelled Tregs or CFSE-labelled Teffs were cultured by themselves or CFSE-labelled Teffs were co-cultured with unlabelled Tregs, or vice versa unlabelled Teffs were co-cultured with CFSE-labelled Tregs at a "physiological" ratio (ratio of Teff/Treg was 10:1) with IL-2 or IL-2 plus TNF. After incubation for 72 h, the proliferation of CFSE labelled cells was analyzed by FACS. The proportion of proliferating cells was indicated in the histogram. The data shown are representative of 3 separate experiments.

contrast, IL-2 or IL-2 plus TNF failed to stimulate the proliferation of Teffs (Fig. 5B).

TNF increased IL-2-induced phosphorylation of Stat5 and immunosuppressive activity of Tregs

It was recently reported that up-regulation of FoxP3 expression and expansion of Tregs by IL-2 involved activation of Stat5 (23). We found that IL-2-stimulated phosphorylation of Stat5 in Tregs was further enhanced by TNF (Fig. 6A). This may be based on TNF up-regulation of CD25 expression, which therefore promotes an IL-2 response by Tregs.

The effect of IL-2 or IL-2 plus TNF on the inhibitory activity of Tregs was examined in co-cultures (72 h) with freshly isolated Teffs. Pre-treatment with IL-2 significantly enhanced the immunosuppressive activity of Tregs (data not shown). Tregs pre-treated

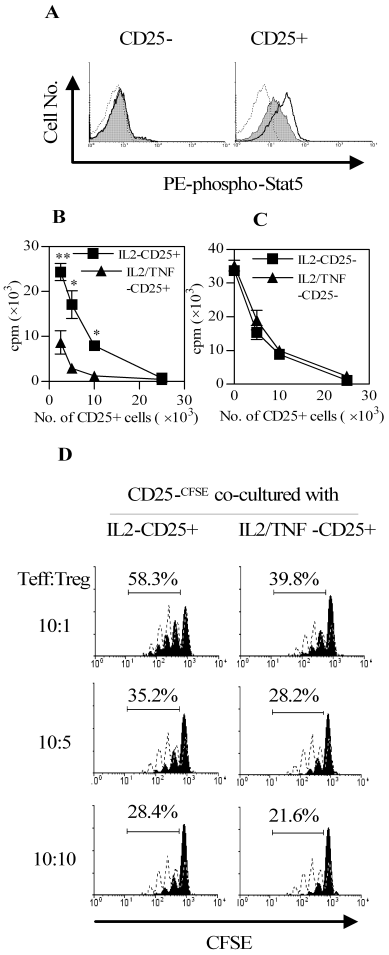

Figure 6. TNF increases IL-2-mediated phosphorylation of Stat5 and enhances function of Tregs. Tregs or Teffs were cultured in the medium supplemented with 10 ng/ml IL-2, with or without 10 ng/ml TNF for 72 h. (A) Intracellular expression of phosphorylated Stat5 was determined by FACS. Dashed line shows isotype control; grey histogram shows cells cultured with IL-2; solid line shows cells cultured with IL-2 plus TNF. (B) Inhibitory activity of IL-2-cultured (IL2-CD25$^+$) or IL-2 plus TNF-pretreated (IL2/TNF-CD25$^+$) Tregs (2.5×10^3 to 2.5×10^4 cells/well) was determined by co-culturing with freshly isolated Teffs (5×10^4 cells/well). The mean cpm of Teffs alone was 66280. (C) The susceptibility of IL-2 cultured (IL2-CD25$^-$) or IL-2 plus TNF-pretreated (IL2/TNF-CD25$^-$) Teffs (5×10^4 cells/well) was examined by co-culture with an increasing number of freshly isolated Tregs (5×10^3 ~2.5×10^4 cells/well). The proliferation was determined after 72 h incubation. Compared with proliferation of IL-2 cultured cells, *P<0.05, **P<0.01. (D) CD45.1$^+$ Teffs were labelled with CFSE and co-cultured with IL-2-, or IL-2 plus TNF-pretreated CD45.2$^+$ Treg at ratio of 10:1, 10:5 and 10:10. The cells were stimulated with CD45.2$^+$ APC and anti-CD3 for 72 h. The proliferation of co-cultured Teffs was analyzed by FACS, gating on CD45.1$^+$ cells (solid histograms). Dashed histograms indicate the proliferation profile of Teffs when they were cultured alone. The results shown are representatives of at least 3 separate experiments.

with IL-2 plus TNF exhibited greater inhibitory activity than Tregs cultured with IL-2 alone ($P<0.01$ to 0.05, Fig. 6B). Since Tregs themselves may proliferate in response to three days of incubation with IL-2 and TNF, we further examined the inhibitory potential of the washed Tregs by co-culturing with CFSE-labelled congenic $CD45.1^+$ Teffs. As shown in Fig 6D, at ratios of Teff to Treg of 10:1, 10:5 and 10:10, the percentage of replicating Teffs were 58.3%, 35.2% and 28.4% when they were co-cultured with IL-2-pretreated Tregs, and was reduced to 39.8%, 28.2% and 21.6% respectively when Teffs were co-cultured with IL-2/TNF-pretreated Tregs. Thus, the CFSE-dilution assay was consistent with [^{3}H] thymidine incorporation assay, and showed that pre-treatment with IL-2 plus TNF enhanced the suppressive activity of Tregs. The effect of these cytokines on the susceptibility of Teffs to inhibition by Tregs was also examined. As shown in Fig 6C, the inhibitory effect of freshly isolated Tregs on Teffs pre-treated with IL-2 or IL-2 plus TNF was similar in potency ($P>0.05$), indicating that after pretreatment with IL-2/TNF, Teffs were still fully susceptible to Treg-mediated suppression. Thus, TNF in combination with IL-2 selectively activated an increased in number of fully functional Tregs.

$TNFR2^{-/-}$ mice subjected to CLP failed to expand Tregs

In order to further evaluate the *in vivo* relevance of our findings, we investigated the

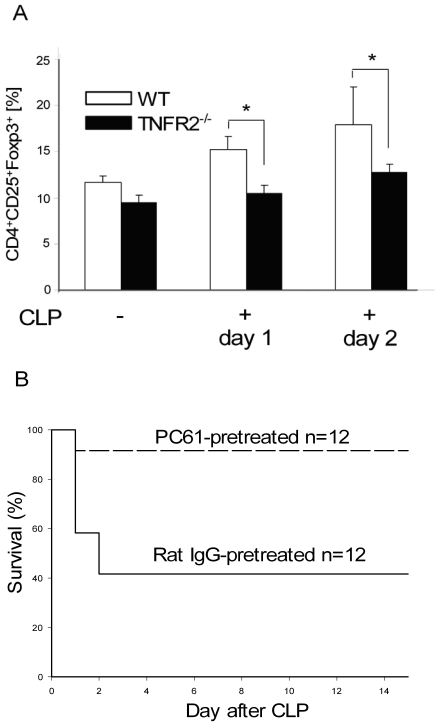

Figure 7. **In $TNFR2^{-/-}$ mice, the proportion of splenic Tregs failed to increase after CLP**. (A) Wild type and $TNFR2^{-/-}$ mice were subjected to sublethal CLP (n = 3 without CLP, n = 6 with CLP for each group). Splenocytes were isolated and the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was determined by FACS from mice without CLP or on day 1 or 2 after CLP. Compared with wild type (wt) mice, * $P < 0.01$. Data shown are representative of 2 separate experiments. (B) Mice (n = 12 per group) were treated with anti-CD25 (PC61) or rat IgG, respectively, 3 days before sublethal CLP operation. Survival was monitored over 15 days and compared. $P = 0.03$. Data shown are representative of 3 separate experiments.

role of Tregs in TNFR2-deficient mice. Since the number and function of Tregs was normal in such mice (data not shown), we studied the effect of challenging mice with CLP, which exhibit a rapid elevation in TNF level (3, 4, 24). In septic patients, the percentage of circulating Tregs is markedly increased, which presumably contributed to the post-septic immunosuppression (25, 26). Consistent with those observations, we found that the number of splenic Tregs significantly increased in wild type mice at 1 and 2 days after CLP. In contrast, Tregs failed to increase in the TNFR2-deficient mice which were subjected to CLP (Fig. 7A). Furthermore, treatment of wild type mice with PC61 antibodies directed against CD25 to deplete Tregs resulted in a remarkable reduction in the early lethality following the CLP procedure (Fig 7B). Our data indicate that *in vivo* expansion of Tregs by interaction of TNF with TNFR2 may play an important role in the induction of post septic immunosuppression, which results in the failure of bacterial clearance and lethality.

Discussion

It was recently reported that ligands for TLR2, but not for TLR4 or TLR9, in conjunction with TCR stimulation induce proliferation of Tregs *in vitro* and *in vivo* (27). This is associated with a transient loss of suppressive activity by the Tregs, which is subsequently restored in an amplified manner. Another group reported TLR2 activation to result in a transient suppression of FoxP3 mRNA expression by mouse Tregs in association with resistance of Teffs to suppression by Tregs by 8-15 h (28). Similarly, our study suggests that short term effects of TNF, as seen in the early phases of inflammatory response, may enable Teffs to expand despite the presence of Tregs, whereas more prolonged exposure to TNF may favor expansion and activation of Tregs. These recent results concerning the effect of TLR2 expression and signalling on the expansion and function of Tregs suggest that during acute infection, pathogen-induced TLR2 activation promote IL-2-dependent Treg proliferation in parallel with temporarily abrogating the suppression of the ongoing immune response by Tregs (27, 28). Since activated T cells are a good source of TNF, it is likely that TNF and TNFR2 participate in these TLR2 responses. The initial transient loss of suppressive activity of Tregs presumably favors elimination of pathogens. Tregs subsequently regain their suppressive capabilities and/or Teffs become more susceptible, thus down-regulate inflammation and contributing to the balance between tolerance and immunity.

The capacity of TNF to stimulate Tregs may underlie the temporal role of TNF in the inflammatory response and account for the opposing effects of TNF in inflammation (29)

and autoimmune diseases (30). For example, wild type mice responded promptly with granuloma formation and hepatosplenomegaly to a microbial agent followed by subsequent resolution of inflammation, while similarly treated $TNF^{-/-}$ mice showed little or no initial response, but subsequently developed a vigorous, disorganized and lethal inflammatory response (29). In experimental autoimmune encephalomyelitis (EAE), $TNF^{-/-}$ mice had a delayed onset of disease; however, once developed, the disease was more severe than in wild-type controls (30). It is possible that in the absence of TNF, Teffs are unable to initiate an effective immune response. However, in the later phase of inflammatory response, deficiency of TNF may result in failure to expand and activate Tregs and cause excessive self destructive inflammation.

TNF has been implicated in the pathogenesis of sepsis for decades as a major mediator of inflammation (1, 2). It is well established that sepsis deeply perturbs immune homeostasis by inducing an initial tremendous systemic inflammatory response that is rapidly followed by immunosuppression (31) and correlated with a fatal outcome (32). Tregs suppress a broad spectrum of immune responses, including dampening host defence against microbial infection (9, 10). Recently, it has been reported that the percentage of circulating Tregs in sepsis-induced immunosuppression is markedly increased which correlates with the progression of sepsis (25, 26). Consistent with these human sepsis studies, we found that the number of Tregs significantly increased in wild type mice, but not in TNFR2-deficient mice, with CLP. Furthermore, we observed that depletion of Tregs prevented CLP-induced mortality. Our data suggest that elevated levels of TNF, by interacting with TNFR2 which predominantly expressed by Tregs, together with the sepsis-induced expansion of Tregs contribute in the post-septic immunosuppression and fatal consequences. In contrast with our observation, a recent report observed that adoptive transfer of in vitro-stimulated Tregs (but not unstimulated Tregs) improved the survival in the mouse CLP model (33). However, as the author indicated, the protective effect of adoptive transferred Tregs was not attributable to the classic immunosuppressive effects of Tregs, because rather than inducing local or systemic immunosuppression, Treg transfer resulted in greater peritoneal cytokine production and mast cells accumulation (33). Their findings may be due to contamination by Teffs, because MACS is subeffecient purification approach.

Our findings that TNF up-regulated FoxP3 expression and immunosuppressive activity of Tregs contrasts with another study of human Tregs which reports that TNF down-regulates FoxP3 expression and blocks suppressive function of Tregs (15). This discrepancy is unlikely due to the species difference of Treg response to TNF, since we observed that TNF in combination with IL-2 also enhanced FoxP3 expression by human

$CD4^+CD25^+$ T cells (data not shown). Valencia et al (15) found that circulating $CD4^+CD25^{hi}$ Tregs incubated with relatively high concentrations of TNF (50 ng/ml) and IL-2 (100 U/ml) lost immunosuppressive activity, probably reflecting only the early transient immunstimulatory phase of TNF.

Our *in vitro* and *in vivo* data are compatible with the observation of Wu and McDevitt's that administration of TNF to adult NOD mice resulted in expansion of Tregs *in vivo* and inhibited diabetes (14). Thus, TNF not only up-regulated FoxP3 and CD25 expression, but also contributed to expanding the Treg pool in mice subjected to an autoimmune or infectious challenge.

In conclusion, TNFR2 preferentially expressed by Tregs endows Tregs with greater capability to use TNF for cellular activation and expansion. Although $TNFR2^{-/-}$ mice have normal numbers of functional Tregs, these fail to expand when challenged by sepsis. Activated T cells are major sources of TNF as well as IL-2 and these two cytokines synergize in promoting the capacity of Tregs to temper both innate and adaptive immune-mediated inflammatory **r**eactions. It is surprising that of the pro-inflammatory cytokine TNF, but not IL-1β or IL-6, up-regulate Tregs. Perhaps this is because TNF is the "master regulator" and is the upstream inducer of the other cytokines (34). The stimulatory effect of TNF mediated by TNFR2 on Treg activity resembles the co-stimulatory effect of TNF on Teffs (16), but is even greater because Tregs express higher levels of TNFR2.

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Chapter 3

Expression of TNFR2 defines a maximally suppressive subset of mouse CD4$^+$CD25$^+$FoxP3$^+$ T regulatory cells: applicability to tumor infiltrating T regulatory cells

Xin Chen, Jeffrey J. Subleski, Heather Kopf, O.M.Zack Howard, Daniela N. Männel and Joost J. Oppenheim

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Abstract

TNFR2 is predominantly expressed by a subset of human and mouse CD4$^+$CD25$^+$FoxP3$^+$ Tregs. In this study, we characterized the phenotype and function of TNFR2$^+$ Tregs in peripheral lymphoid tissues of normal and tumor bearing C57BL/6 mice. We found that TNFR2 was expressed on 30%-40% of peripheral activated/memory subset of Tregs that were most highly suppressive. In contrast, TNFR2$^-$ Tregs exhibited the phenotype of naïve cells and only had minimal suppressive activity. Although not typically considered to be Tregs, CD4$^+$CD25$^-$TNFR2$^+$ cells nevertheless possessed moderate suppressive activity. Strikingly, the suppressive activity of TNFR2$^+$ Tregs was considerably more potent than that of reportedly highly suppressive CD103$^+$ Tregs. In the Lewis lung carcinoma model, more highly suppressive TNFR2$^+$ Tregs accumulated intratumorally than in the periphery. Thus, TNFR2 identifies a unique subset of mouse Tregs with an activated/memory phenotype and maximal suppressive activity which may account for tumor-infiltrating lymphocyte mediated immune evasion by tumor.

Introduction

TNF is a pleiotropic cytokine that is a major participant in the initiation and orchestration of inflammation and immunity (1). TNF is also implicated in inflammation-associated cancers, produced either by tumor cells and/or by infiltrating leukocytes (2). TNF mediates its biological functions through its receptors: TNFR1 and TNFR2. TNFR1 through its death domain mediates the cytotoxic effect while TNFR2 is largely confined to cells of the immune system and its biological role is still not fully understood (1). TNF has been shown to have proinflammatory effects, nevertheless, increasing evidence reveals TNF also has unexpected immunosuppressive effects and this may be based on the capacity of TNF in concert with IL-2 to activate and expand mouse CD4$^+$CD25$^+$ T regulatory cells (Tregs) (3), presumably by interacting with TNFR2 which is preferentially expressed by both human and mouse Tregs (3, 4). Thus, up-regulation of Treg activity by TNF may mediate the delayed immunosuppressive effect of TNF on inflammatory responses and the expansion of Tregs in the tumor microenvironment. Consequently, we hypothesized that TNFR2 expression may identify the more functionally active Tregs in tumor-infiltrating lymphocytes (TILs).

Materials and Methods

Mice, cells and reagents. Female wild type C57BL/6 mice, 8 to 12 wk old, were provided by the Animal Production Area of the NCI (Frederick, MD). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.). C57BL/6-derived Lewis lung carcinoma (LLC) cell line was obtained from ATCC. Antibodies purchased from BD Biosciences (San Diego, CA) consisted of anti-CD3 (145-2C11), CD4 (GK1.5), anti-CD25 (PC61), CD45RB (16A), CD62L (MEL-14), CD44 (IM4), CD69 (H1.2F3), CD103 (M290), TNFR2 (TR75-89), CTLA4 (UC10-4F10-11) and CD16/CD32 (2.4G2). Anti CD3e (145-2C11), Foxp3 Staining Set (FJK-16s), GITR (DTA-1) Abs were purchased from eBioscience (San Diego, CA).

Mouse tumor inoculation and separation of tumor infiltrating lymphocytes (TILs). C57BL/6 mice were inoculated subcutaneously in the right rear flank with 100,000 LLC cells. After two weeks, tumors were excised, minced and digested in RPMI 1640 supplemented with 1 mg/ml collagenase IV, 0.1 mg/ml DNase I.

Cell purification, in vitro culture and proliferation assay. CD4 subsets were purified from spleen and lymph nodes (inguinal, axillary and mesenteric regions) or TILs using Cytomation MoFlo cytometer (Fort Collins, CO), yielding a purity of ~98% for both subsets. For in vitro assays of inhibition of proliferation by Treg, CFSE-labelled (2 μM, 8 min at room temperature) responder cells (5×10^4 cells/well) were seeded in a U-bottom 96-well plate with 2×10^5 cells/well of APCs (T cell-depleted, irradiated spleen cells) plus 0.5 μg/ml of functional grade anti-CD3e antibody. Subsets of CD4 cells were added to the wells at the desired ratio. After 48 h, CFSE dilution was determined with FACS.

Flow Cytometry. After blocking FcR, cells were incubated with appropriately diluted antibodies. Data were acquired on a FACSort (BD Biosciences, Mountain View, CA) and data analysis was conducted using CellQuest software (BD Biosciences).

Results and Discussion

Phenotype and distribution of mouse TNFR2$^+$ Tregs

As shown for normal C57BL/6 (B6) mice in Fig 1A, the expression of TNFR2 was largely restricted to splenic CD4$^+$ cells. Similar results were observed by LN cells (data not shown). CD4$^+$CD25$^+$ T cells were the primary cell type expressing TNFR2, while fewer CD4$^+$CD25$^-$ T cells expressed this receptor (Fig 1B). The proportions of CD25$^+$TNFR2$^+$ cells, CD25$^+$TNFR2$^-$ cells and CD25$^-$TNFR2$^+$ splenic CD4 cells were 3.6%, 6.1% and 2.8%, respectively (Fig 1C).

Tregs are heterogeneous and consist of activated/memory as well as naïve cells. As shown in Fig 1D, TNFR2$^+$ Treg cells were CD45RB^{lo}, CD62L^{lo}, CD44^{hi} and expressed relatively high levels of CD69, CD103, GITR and CTLA-4, indicative of the memory and activated phenotype. In contrast, naïve Tregs were CD45RB^{hi}, CD62L^{hi}, CD44^{lo} and expressed relative low levels of CD69, CD103, GITR and CTLA-4, and were TNFR2$^-$. CD4$^+$CD25$^-$ TNFR2$^+$ cells contained fewer CD45RB^{hi} and CD62L^{hi}, and more CD44^{hi} and CD69$^+$ cells, compared with CD4$^+$CD25$^+$TNFR2$^-$ cells. Furthermore, TNFR2 showed a closer relationship than CD25 to the subset of memory and activated mouse peripheral FoxP3$^+$ Treg cells. Both TNFR2$^+$ and TNFR2$^-$ subsets of CD4$^+$CD25$^+$ cells expressed comparably high levels of FoxP3. However, the intensity of FoxP3 expression was consistently higher in CD4$^+$CD25$^+$TNFR2$^+$ cells than in CD4$^+$CD25$^+$TNFR2$^-$ cells. Although only about 1% of CD4$^+$CD25$^-$TNFR2$^-$ cells were FoxP3$^+$ cells, 25~40% of CD4$^+$CD25$^-$TNFR2$^+$ cells expressed FoxP3 (Fig 1D).

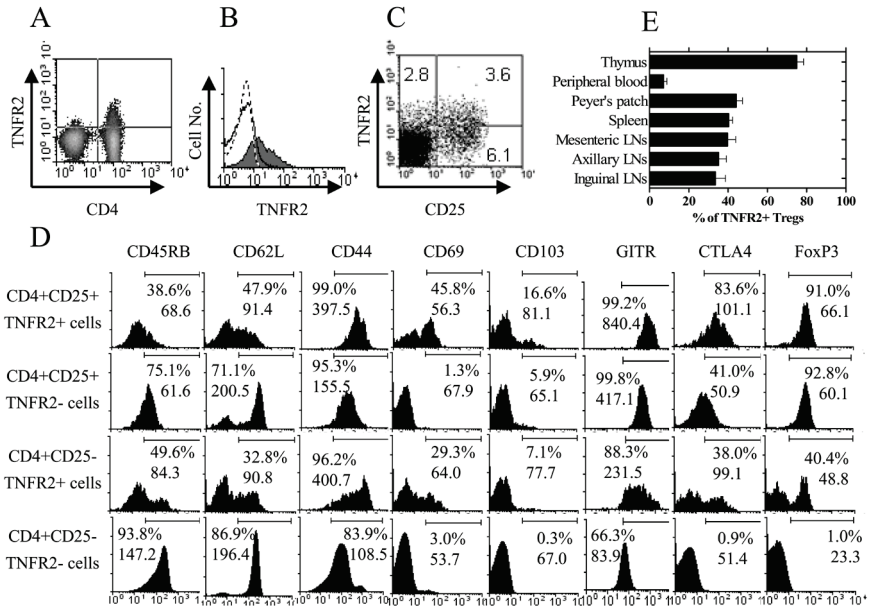

Figure 1. Phenotypic characterization and distribution of normal C57BL/6 mouse CD4$^+$CD25$^+$TNFR2$^+$ Tregs. (A) Splenocytes were stained with anti CD4 and TNFR2 Abs and analyzed by FACS. (B) Splenocytes were stained with anti CD3, CD4, CD25 and TNFR2 Abs. The expression of TNFR2 by CD4$^+$CD25$^+$ cells (grey histogram) and CD4$^+$CD25$^-$ cells (solid line histogram) was analyzed by FACS, gating on CD3$^+$ cells. Dashed line represents isotype control. (C) Splenocytes were stained with anti CD3, CD4, CD25 and TNFR2. Expression of CD25 and TNFR2 was analyzed with FACS by gating on CD3$^+$CD4$^+$ cells. (D) Splenocytes were stained with anti CD4, CD25, TNFR2 and CD45RB, or CD62L, or CD44, or CD69, or CD103, or GITR, or CTLA-4, or FoxP3 Abs. CTLA-4 and FoxP3 were stained intracellularly. Expression of phenotypic markers was analyzed with FACS by gating on indicated CD4 subsets. (E) Cells from indicated lymphoid tissues and peripheral blood were stained with anti CD3 (or CD8 for thymocytes), CD4, CD25 and TNFR2 Abs. The percentage of TNFR2$^+$ cells was analyzed with FACS by gating on CD3$^+$CD4$^+$CD25$^+$ population (except for thymocytes which were gated on CD8$^-$CD4$^+$CD25$^+$ cells). Error bars indicate SE derived from 3 mice (n=3). Numbers in the quadrants represent percentage of positive staining cells (%).The numbers in the histograms show percentage of positive cells (%) and MFI. Data shown are representatives of at least 3 separate experiments with similar results.

In normal C57BL/6 mice, ~80% of CD8$^-$CD4$^+$CD25$^+$ thymocytes were TNFR2$^+$, which resembles the reported high TNFR2 expression by human thymic Tregs (4). In peripheral LNs, spleen and Peyer's patches, 30%~40% of CD4$^+$CD25$^+$ cells expressed TNFR2. In

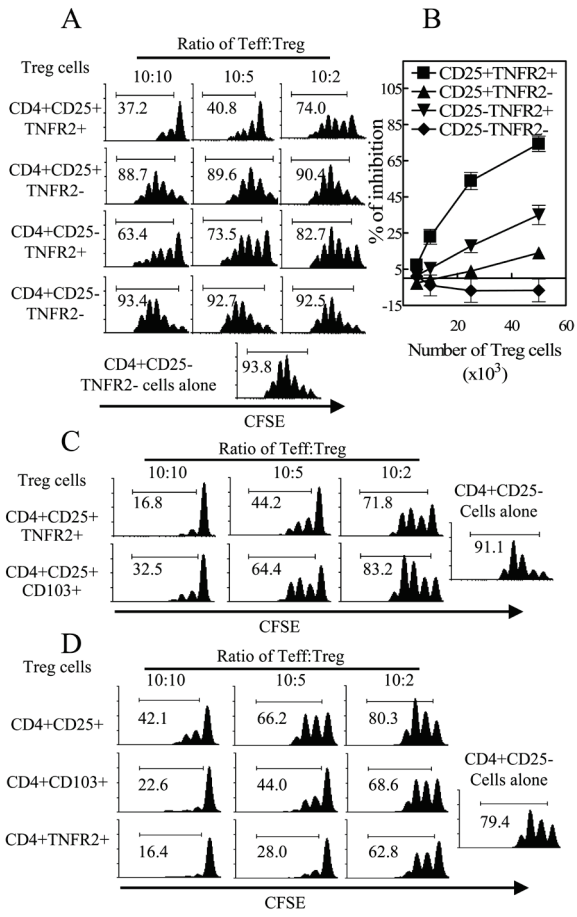

Figure 2. CD4$^+$CD25$^+$TNFR2$^+$ T cells were highly potent suppressor cells. Flow-sorted CD4$^+$CD25$^-$TNFR2$^-$ or CD4$^+$CD25$^-$ T cells (5×10^4 cells/well) were labeled with CFSE and cultured alone or co-cultured with indicated number or ratio of flow-sorted CD4 subsets from spleen and LNs of normal C57BL/6 mice. The percentage of CFSE-diluted cells was shown in the histograms. (A), (C) and (D) show a representative data of at least 3 separate experiments with similar results and (B) shows summary of percent inhibition of replication (%) from 6 separate experiments.

contrast, in mouse peripheral blood, less than 10% of CD4$^+$CD25$^+$ cells were TNFR2$^+$ (Fig 1E).

Mouse TNFR2$^+$ Tregs are the most highly suppressive cells

Upon stimulation with APC and anti-CD3, only CD4$^+$CD25$^-$TNFR2$^-$ T cells were readily activated to proliferate and produce IFNγ, whereas CD4$^+$CD25$^+$TNFR2$^+$ and CD4$^+$CD25$^+$TNFR2$^-$ cells were unreactive. Interestingly, TNFR2-expressing CD4$^+$CD25$^-$ T cells were also non-responsive to TCR stimulation (data not shown).

Next, we examined the suppressive potential of these CD4 subsets. Flow-sorted CD4 subsets were co-cultured as shown in Fig 2A. As we expected, CD4$^+$CD25$^+$TNFR2$^+$ T cells were potent suppressor cells and inhibited replication of responder CD4$^+$CD25$^-$TNFR2$^-$ T cells. Surprisingly, CD4$^+$CD25$^+$TNFR2$^-$, comprising the majority of classical Tregs (60-

70% of CD4$^+$CD25$^+$FoxP3$^+$ T cells), had only minimal suppressive activity. Although not usually considered to be Tregs, TNFR2-expressing CD4$^+$CD25$^-$ T cells had moderate and consistently more potent suppressive capacity than CD4$^+$CD25$^+$TNFR2$^-$ cells. Due to technical difficulty of sorting FoxP3$^+$ cells from normal C57BL/6 mice, we have not examined whether FoxP3$^+$ cells present in CD4$^+$CD25$^-$TNFR2$^+$ subset are solely responsible for the suppressive activity. We very carefully repeated this experiment and obtained highly consistent results (Fig 2B). Furthermore, Balb/c mouse TNFR2+ Tregs also had a memory/activated phenotype and were upto10 fold more suppressive than TNFR2- Tregs (data not shown).

TNFR2 is a better phenotypic marker of highly suppressive cells than CD103

It has been reported that CD103 expression can define the most potent suppressive subset of CD4$^+$CD25$^+$ Tregs (5). Since CD4$^+$CD25$^+$TNFR2$^+$ T cells expressed the highest level of CD103, it is possible that the expression of CD103 may more accurately predict the suppressive potential of CD4 subsets. We confirmed the previous report (5) that CD4$^+$CD25$^+$CD103$^+$ T cells were more potent suppressors than CD4$^+$CD25$^+$CD103$^-$ T cells (data not shown). However, the inhibition of proliferation by CD103$^+$ Tregs was significantly less potent than by TNFR2$^+$ Tregs, as shown by both CFSE-dilution assay (Fig 2C) and [^{3}H] thymidine incorporation assay and also in the inhibition of cytokine production ($P<0.01\sim0.05$, data not shown). Furthermore, CD4$^+$TNFR2$^+$ T cells exhibited more suppressive effects than CD4$^+$CD103$^+$ T cells, and both of these subsets were more suppressive than the more heterogeneous CD4$^+$CD25$^+$ T cells (Fig 2D). Consequently, TNFR2 is superior to both CD103 and CD25 in defining functional CD4 T suppressor cells.

Tumor infiltrating Tregs express higher TNFR2 level and are more suppressive

Tregs accumulate intratumorally and promote tumor growth by inhibiting anti- tumor immune response (6). Based on reports that TNFR2 is expressed by TILs isolated from several human solid tumors (7), we hypothesized that TNFR2$^+$ Tregs with enhanced suppressive potential may be more prevalent in the tumor microenvironment. For this purpose, we examined the C57BL/6 mouse Lewis lung carcinoma (LLC) tumor model. As predicted, 75~100% of CD4$^+$CD25$^+$ TILs expressed TNFR2. The proportion of TNFR2$^+$ cells present in the subset of CD4$^+$CD25$^-$ TILs was also increased over the low levels present in splenic and LN cells (Fig 3A). Similar results were also observed in the Balb/c mouse 4T1 breast tumor model (data not shown).

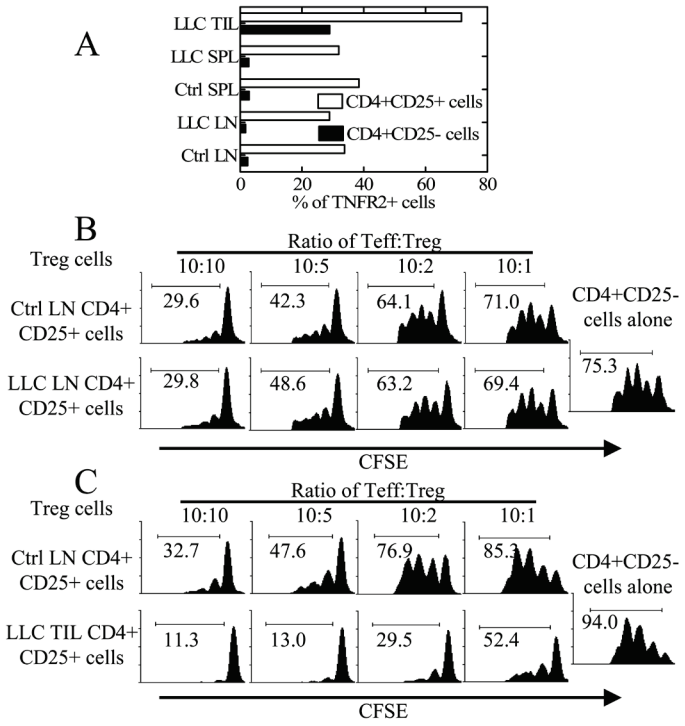

Figure 3. Highly suppressive TNFR2$^+$ Tregs accumulated in the TILs of LLC mouse model. (A) LN cells, spleen cells (SPL) and TILs from LLC tumor bearing mice were stained with CD4, CD25 and TNFR2. Expression of TNFR2 was analyzed with FACS by gating on CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells from LLC bearing mouse TIL (LLC TIL) or spleen (LLC SPL) or LNs (LLC LN) or from tumor free C57BL/6 mouse spleen (Ctrl SPL) or LNs (Ctrl LN). (B-C) CD4$^+$CD25$^-$ T cells from normal control mouse LNs (5×10^4 cells/well) were labeled with CFSE and cultured alone or co-cultured with indicated source of Tregs at desired ration. The percentage of CFSE-diluted cells was shown in the histograms. Data shown are representatives of 3 separate experiments with similar results.

We next tested the prediction that the inhibitory activity of LLC CD4$^+$CD25$^+$ TILs should be greater than that of peripheral LN CD4$^+$CD25$^+$ cells. As shown in Fig 3B, there was no difference in the suppressive potency of Tregs over various ratios of Teff:Treg from peripheral LNs of tumor bearing and control mice. In contrast, TIL CD4$^+$CD25$^+$ cells retrieved from LLC tumors exhibited far more potent suppressive activity than LN CD4$^+$CD25$^+$ cells from tumor-free mice (Fig 3C). Thus, the suppressive effects of peripheral LN Tregs and TIL Tregs correlated well with their level of TNFR2 expression. As shown in Fig. 3B-C, the potent inhibitory effect exerted by CD4$^+$CD25$^+$ TILs was not

antigen specific, since both targeted responder cells and APCs were from tumor-free mice. The phenotype of TIL CD4$^+$CD25$^+$ cells, which were 75~100% TNFR2$^+$, resembled that of normal mouse LN TNFR2$^+$ Tregs and were indicative of an activated/memory subset (data not shown).

Although other factors in the tumor microenvironment may also contribute to the activation of TIL Tregs, the presence of TNF in the inflammatory tumor site is most likely to contribute to up-regulating the number of functional Tregs by activating TNFR2 (3). It has been reported that TGFβ, a cytokine crucial for *de novo* generation of Tregs, is also able to induce TNF and TNFR2 expression on CD4 cells (8) and may therefore be responsible for the increase of TNFR2$^+$ Tregs in the tumor. TNFR2 has also been reported to be a co-stimulator for antigen-driven T cell responses (1), thus, this receptor can also serve as a co-stimulator for the response of Treg to tumor antigen. Indeed, it has been shown that anti-TNF as well as anti-TNFR2 antibodies can inhibit proliferation of TNFR2-expressing TILs isolated from human solid tumors (7). Despite reports that anti-TNF therapy increases Treg activity in rheumatoid arthritis (9), our preliminary observations also indicate that anti-TNF therapy actually decreased the proportion of FoxP3+ Tregs in TILs of various mouse tumor models (our unpublished data). Thus blockade of TNF-TNFR2 interaction may result in down-regulation of Treg activity and this may provide the mechanistic basis for the reported therapeutic efficacy of anti-TNF therapy in cancer (2).

Several lines of evidence suggest that immunosuppressive action of TNF is mediated by TNFR2. For example, in experimental autoimmune encephalomyelitis (EAE) mouse model, TNFR1 deficient mice were completely resistant to induction of disease, while TNFR2 deficient mice exhibited more severe EAE (10). The immunosuppressive action of TNFR2-dependent effects of TNF is most compatible with our observations that TNF is a potent and selective activator of Tregs (3).

It was reported that CD103 expression defines a potent suppressive subset of Tregs (5) and CCR6 expression defines an effector/memory-like subset of Tregs (11). Expression of CD103 partially correlated with TNFR2 expression by Tregs (Fig. 1D), however, the reported low thymic level of CD103$^+$ Tregs (5) as well as high level in peripheral blood of CCR6$^+$ Tregs (11) is distinct from TNFR2$^+$ Tregs in distribution. Furthermore, CD103 (integrin αEβ7) serves as a homing receptor for mucosa-seeking populations and has no clear relationship to the suppressive function of Tregs (5). In addition, while CD103 is only expressed by mouse Tregs, TNFR2 is also expressed by human Tregs (4) and correlates with their greater suppressive effects (our unpublished data). CCR6 is expressed by both

memory type Tregs as well as T effector cells and serves to direct trafficking of CCR6$^+$ cells to inflammatory sites. Although CCR6$^+$ Tregs were described as effector/memory-like cells, CCR6$^+$ Tregs and CCR6$^-$ Tregs do not show any differences in suppressive activity (11). Unlike CD103 and CCR6, TNFR2 is a functional receptor for TNF and therefore is likely to respond to TNF by activating and expanding Tregs at inflammatory sites (3). Nevertheless, TNFR2 knockout mice have a normal functional CD4$^+$CD25$^+$ Tregs. Thus TNFR2 is not necessary to maintain Treg activity, but it may play a crucial role in the activation and expansion of Tregs at inflammatory or tumor sites. Presumably this function of TNFR2 reflects down-regulatory effect of TNF, but not IL-1 and IL-6, on the inflammatory response (3).

Foxp3 is reported as both a specific marker and commitment factor for the Treg cell lineage (6). Both TNFR2$^+$ and TNFR2$^-$ CD4$^+$CD25$^+$ T cells from normal C57BL/6 mice expressed comparable high level of FoxP3. Nevertheless, the suppressive function of FoxP3$^+$TNFR2$^-$ cells is not appropriately "turned on" or is intrinsically deficient. Furthermore, CD4$^+$CD25$^-$ TNFR2$^+$ cells were more suppressive than CD4$^+$CD25$^+$TNFR2$^-$ cells, even though the former contained much less FoxP3$^+$ cells than the latter. Thus, FoxP3 expression by resting mouse CD4 cells may not be sufficient to confer suppressive capacity. Our data suggest that expression of TNFR2 is more relevant to suppressive phenotype than expression of CD25 and even FoxP3.

Apparently, TGFβ and IL-10 did not contribute to the in vitro suppressive activity of TNFR2$^+$ Tregs, because neutralizing antibodies against TGFβ and IL-10 failed to attenuate activity of TNFR2$^+$ Treg (data not shown). Soluble TNFR2 potentially shed by TNFR2$^+$ Tregs is also unlikely to influence Treg activity, since the level of soluble TNFR2 in the supernatant of co-cultures containing TNFR2$^+$ Tregs was not increased in our in vitro Treg function assay (48 h, data not shown). CTLA4 expression is only detected in the Treg compartment of CD4 cells in normal mice and its expression is implicated in the suppressive function of Tregs (6). This suppressive molecule was expressed by >80% of TNFR2$^+$ Tregs, which is in agreement with the potent suppressive capacity of this subset of Tregs. Intriguingly, the superior suppressive activity of CD4$^+$CD25$^-$TNFR2$^+$ cells over CD4$^+$CD25$^+$TNFR2$^-$ cells correlate directly with the intensity of CTLA4 expression by these two subsets (Fig 1D). TNFR2 just like CTLA4 is selectively expressed by functional Tregs rather than Teffs, even in the absence of CD25 and FoxP3.

Taking together, our data demonstrate that TNFR2 expression defines a unique subset of mouse Tregs with an activated/memory phenotype and highly potent suppressive activity.

In mouse tumor models, the proportion of tumor infiltrating suppressive TNFR2$^+$ Tregs was dramatically increased, suggesting therapeutic value by targeting TNFR2$^+$ Tregs in tumor immunotherapy.

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Chapter 4

Co-expression of TNFR2 and CD25 identifies more of the functional $CD4^+FoxP3^+$ regulatory T cells in human peripheral blood

Xin Chen, Jeffrey J. Subleski, Ryoko Hamano, O.M.Zack Howard, Robert H. Wiltrout and Joost J. Oppenheim

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Summary

Previously we found that co-expression of CD25 and TNFR2 identified the most suppressive subset of mouse regulatory T cells (Tregs). Here, we report that human peripheral blood (PB) FoxP3^{+} cells present in CD25^{high}, CD25^{low} and even CD25^{-} subsets of CD4 cells expressed high levels of TNFR2. Consequently, TNFR2-expressing CD4^{+}CD25^{+} Tregs included all of FoxP3^{+} cells present in CD4^{+}CD25^{high} subset as well as a substantial proportion of FoxP3^{+} cells present in CD4^{+}CD25^{low} subset. CD4^{+}CD25^{+}TNFR2^{+} cells identified 5-fold greater number of PB CD4 lymphocytes as Tregs than identified by CD4^{+}CD25^{high} cells, and expressed comparable levels of FoxP3^{+} cells as reported CD4^{+}CD25^{+}CD127$^{low/-}$ Tregs. Furthermore, this population of cells exhibited the characteristic Treg phenotype, including expression of high levels of CTLA-4, CD45RO, CCR4 and low levels of CD45RA and CD127. Upon TCR stimulation, human PB CD4^{+}CD25^{+}TNFR2^{+} cells were anergic and markedly inhibited the proliferation and cytokine production of co-cultured T responder cells. In contrast, CD4^{+}CD25^{+}TNFR2^{-} and CD4^{+}CD25^{-}TNFR2^{+} T cells did not show inhibitory activity. Since some non-Tregs express TNFR2, the combination of CD25 and TNFR2 must be used to identify larger population of human Tregs, which may prove to be of diagnostic and therapeutic benefit in cancer and autoimmune diseases.

Introduction

Identification of CD25-expressing CD4 cells in Balb/c mice as regulatory T cells (Tregs) has greatly advanced our understanding of the basic processes that control immune tolerance (1). These suppressor cells, comprising 5-10% of mouse peripheral $CD4^+$ T cells and expressing the X chromosome-encoded forkhead transcription factor, FoxP3, play an important role in preventing immunopathology by suppressing immune responses to autoantigens, however, they also attenuate natural immune responses against tumor antigens (2, 3). Tregs are a good target for therapeutic manipulation to induce or abrogate immunological tolerance to self and non-self antigens (4, 5).

The quantitative identification and enrichment of viable Treg cells requires reliable surface markers that are selectively expressed on Tregs. Utilization of CD25 to define human Treg is problematic since this IL-2 receptor α chain does not discriminate regulatory from antigen-responsive activated effector T cells (Teffs). Only ~1% of normal human circulating CD4 cells that express the highest levels of CD25, termed $CD25^{high}$, reliably exhibit suppressive activity (6, 7) and have high levels of FoxP3 expression (8, 9). Although the identification of $CD25^{high}$ as a marker for human Tregs by Baecher-Allan/Hafler and colleagues has greatly advanced in the purification of viable human Tregs (6, 7), it is difficult to accurately quantitate Treg cells based on $CD25^{high}$ and this criterion may underestimate Treg levels. In addition, the low frequency of $CD25^{high}$ makes it difficult to isolate sufficient number of Tregs for either in vitro study or for in vivo cellular therapy. Unfortunately, FoxP3 and CTLA-4 expressed by Tregs become detectable only when cells are fixed and permeabilized, and thus can not be used as a biomarker to isolate viable cells. Other reported markers of human Tregs, although helpful in the further characterizing Tregs, also have their limitations when they are used to define human Tregs. For example, HLA-DR expressing Tregs represent an even lower percentage (often <1%) of $CD4^+$ T cells (10). CD27 has been proposed as a surface marker of human Tregs (11), however, the vast majority of PB antigen-activated $CD25^+$ effector T cells (Teffs) also express high levels of CD27 (12). Recently, major progress in the identification of biomarker of Tregs has been made by Liu/Bluestone and Seddiki/Fazekas de St Groth and their colleagues who reported that human Tregs expressed low levels of CD127, the IL-7 receptor α chain, and that the absence of this molecule therefore can be used with CD25 to define human Tregs (9, 13). Nevertheless, an additional surface marker which positively correlates with FoxP3

expression and immunosuppressive function may improve the identification and isolation of human Tregs.

It has been shown that human thymic $CD4^+CD25^+$ Tregs constitutively express TNFR2, while thymic $CD4^+CD25^-$ cells do not express this receptor (14). We found that the majority (~80%) of mouse thymic Tregs ($CD8^-CD4^+CD25^+$) are also TNFR2-expressing cells (15). Normally 30~40% of $CD4^+CD25^+$ cells comprised greater than 90% $FoxP3^+$ cells in peripheral lymphoid tissues of unstimulated Balb/c mice and C57BL/6 (B6) mice expressed TNFR2, while only <10% of $CD4^+CD25^-$ Teffs were $TNFR2^+$ cells and up to 40% of them expressed FoxP3 (15, 16). Thus, TNFR2 is predominantly expressed on mouse as well as on human Tregs (14, 16). Furthermore, TNFR2 expression identified the most potent suppressive subset of mouse $CD4^+CD25^+$ Tregs, while $CD4^+CD25^+TNFR2^-$ T cells in normal C57BL/6 mice have only minimal or no suppressive activity (15). The findings concerning mouse Tregs could often be extrapolated to human. For example, CD25 as a mouse CD4 Treg marker led to the discovery that $CD25^{high}$ could define human CD4 Tregs (6). Recently, it was reported that human $CD8^+CXCR3^+$ cells were actually the counterpart of mouse $CD8^+CD122^+CXCR3^+$ Tregs (17). We therefore hypothesized that the expression of TNFR2 might help identify functional human suppressor cells. In this report, we show that human PB $CD4^+CD25^+TNFR2^+$ T cells also consistently exhibited the phenotypic and functional attributes of Tregs.

Materials and Methods

Cells and reagents. Human peripheral blood enriched in mononuclear cells was obtained from normal donors by leukapheresis (Transfusion Medicine Department, Clinical Center, National Institutes of Health, Bethesda, MD, with an approved human subjects agreement). The blood was centrifuged through Ficoll-Hypaque (Sigma), and PBMCs collected at the interface were washed with PBS and centrifuged through isoosmotic Percoll (Pharmacia, Uppsala, Sweden) gradient. CD4-FITC/-APC, CD25-PE/-PE-Cy5/-APC, CD152 (CTLA-4)-APC, CD45RO-PE-Cy5, CCR4-biotin, CD120b (TNFR2)-biotin, purified CD3 and various fluorochrome conjugated streptavidin were from BD Pharmingen (San Diego, CA). CD45RA-PerCP Cy5.5, CD127-PerCP Cy5.5, HLA DR-PerCP Cy5.5 and FoxP3-APC staining set were from eBioscience (San Diego, CA). CD120b (TNFR2, MR2-1)-PE was from Serotec (Raleigh, NC). CCR7-PE was from R&D systems, Inc. (Minneapolis, MN).

Cell purification, in vitro cell culture and proliferation assay. $CD4^+$ cells were purified from freshly isolated human PBMCs with human CD4 microbeads and LS column

(Miltenyi Biotec Inc., Auburn, CA). $CD4^+CD25^+TNFR2^+$, $CD4^+CD25^+TNFR2^-$, $CD4^+CD25^-TNFR2^+$ and $CD4^+CD2^-TNFR2^-$ cells were purified from $CD4^+$ cells using Cytomation MoFlo cytometer (Fort Collins, CO), yielding a purity of ~98% for all subsets. Autologous PBMCs were used as APCs by depletion of $CD4^+$ cells with anti-human CD4 microbeads (Miltenyi Biotec Inc.). APCs were irradiated with 4,000 R.

For in vitro assays of inhibition of proliferation by Treg, $CD4^+CD2^-TNFR2^-$ cells ($2.5\sim5\times10^4$ cells/well) were seeded in a U-bottom 96-well plate in medium [RPMI 1640 with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, 10 mM HEPES]. They were co-cultured with 2×10^5 /well of APCs and 0.5 μg/ml of soluble anti human CD3 Ab. $CD4^+$ subsets were added to the wells at a desired ratio to $CD4^+CD25^-TNFR2^-$ cells. Cells were pulsed with 1 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) per well for the last 6 h of a three day culture period. For CFSE dilution assay, CFSE-labelled (2 μM, 8 min at room temperature) responder cells (MACS-purified autologous CD4 cells, 5×10^4 cells/well) were seeded in a U-bottom 96-well plate together with 2×10^5 cells/well of APCs and 0.5 μg/ml of anti-CD3 antibody. FACS-purified $CD4^+CD25^+TNFR2^+$ cells or other CD4 subsets were added to the wells at the desired ratio. After 48~72 h, CFSE dilution was determined with FACS. In some experiments, IFNγ levels in the supernatants of cultures were determined by SearchLight Human Cytokine Array (Pierce Biotechnology, Woburn, MA).

Flow Cytometry. After blocking FcR, cells were incubated with appropriately diluted antibodies. Data were acquired on a FACSort (BD Biosciences, Mountain View, CA) and analysis was conducted using CellQuest software (BD Biosciences).

Statistical analysis. Comparisons of two groups of data were analyzed by two-tailed Student's *t* test using Graphpad Prism 4.0.

Results

Relationship between expression of TNFR2 and FoxP3 on human PB CD4 cells

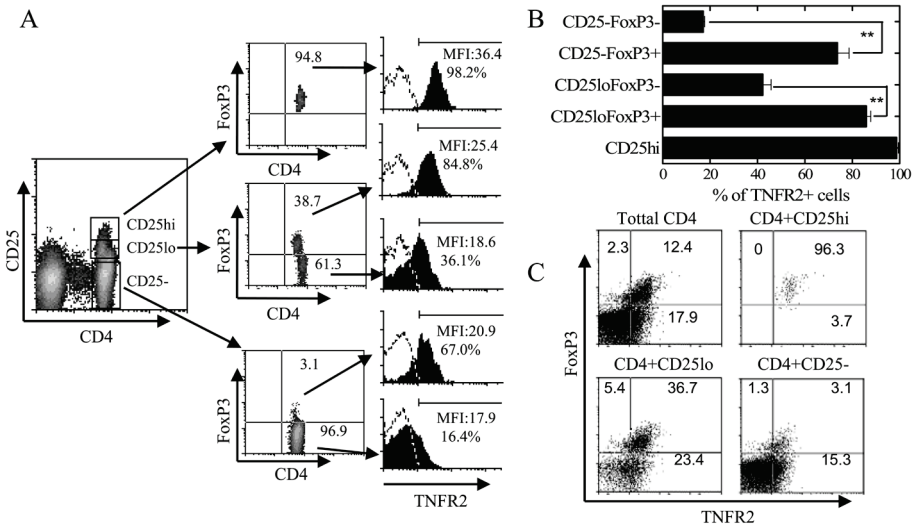

Figure 1. Human PB FoxP3$^+$ Tregs express high levels of TNFR2. Freshly isolated human PBMCs were stained for CD4, CD25 and TNFR2 and then were fixed and stained intracellularly for FoxP3. CD4 cells were analyzed by FACS, gating on lymphocytes via their forward and side scatter properties and CD4 staining. (A) FoxP3 and TNFR2 expression by CD25^{hi}, CD25^{lo} and CD25$^-$ subsets of CD4 cells. (B) Percentage of TNFR2-expressing cells in CD4 subsets. (C) FoxP3 and TNFR2 expression by total CD4 cells, CD4$^+$CD25^{hi} cells, CD4$^+$CD25^{lo} cells and CD4$^+$CD25$^-$ cells. Date shown in (A) and (C) are representative of at least three separate experiments on different donors with similar results. Data shown in (B) are summarized from three different donors (mean ± SEM, N=3). The numbers in the quadrants indicate the percentage of positive cells. The numbers in histograms are mean fluorescence intensity (MFI) and percentage of positive cells (%). Dashed line histogram shows isotype control. Comparison of TNFR2 expression on CD25^{lo}FoxP3$^+$ and CD25^{lo}FoxP3$^-$ or CD25$^-$FoxP3$^-$ and CD25$^-$FoxP3$^+$ cells: ** P<0.01.

It was recently reported that TNFR2 expression was highest on human peripheral blood (PB) CD4$^+$CD25^{high} T cells, but could also be detected on CD4$^+$CD25^{int} and on a minor fraction of CD4$^+$CD25$^-$ T cells (18). We confirmed this observation and further examined the relationship between TNFR2 and FoxP3 expression in human PB CD4 subsets with different CD25 expression levels, since to date, FoxP3 remains the most specific marker of the Treg lineage (3). Normal human peripheral lymphocytes contained ~40% of CD4 cells and ~12% of CD25$^+$ cells (data not shown). About 1% of the brightest CD25-expressing CD4 cells were defined as CD25^{high} (or CD25^{hi}) cells (6). For convenience sake, all CD25$^+$ cells except for the CD25^{high} were defined as CD25^{low} (or CD25^{lo}) cells. Consistent with the report that human PB CD4 cells with high levels of CD25 have repeatedly been shown to be functional Tregs (6), most of the CD25^{high} cells were FoxP3$^+$ and almost all of them

(97.4~100%) expressed the highest levels of TNFR2 (Fig 1A). We have found that although usually not considered to be Tregs, human PB $CD4^+CD25^{low}$ cells still consisted of about 40% of $FoxP3^+$ cells. From 83.3~89.6% of $FoxP3^+$ cells in this $CD25^{low}$ subset were $TNFR2^+$ cells while, in contrast, only 36.1~48.4% of $FoxP3^-$ presumably effector T cells (Teffs) in this population expressed TNFR2. Furthermore, a small but detectable 3~5% of $CD25^-$ cells also expressed FoxP3. 67~83.3% of $CD25^-FoxP3^+$ cells did express TNFR2, while only 16.0~17.9% of $CD25^-FoxP3^-$ cells expressed low levels of TNFR2 per cell (Fig 1A). The high levels of TNFR2 expression on $FoxP3^+$ cell, regardless CD25 expression, were consistently observed in this study. The percentage of $TNFR2^+$ cells present in $CD25^{low}FoxP3^+$ and $CD25^-FoxP3^+$ subsets was also markedly higher than in the $CD25^{low}FoxP3^-$ and $CD25^-FoxP3^-$ subsets, respectively ($P<0.01$, Fig 1B). Thus TNFR2 and FoxP3 expression in CD4 subsets were directly correlated (Fig 1C). Approximately 85% of PB $CD4^+FoxP3^+$ cells expressed high levels of TNFR2, in contrast, only ~20% of $FoxP3^-$ cells expressed low levels of TNFR2. Therefore, both the proportion of cells and the level of expression (MFI) of TNFR2 are highly correlated with FoxP3 expression in human PB CD4 cells.

Relationship of combination of CD25 and TNFR2 expression in PB CD4 cells to FoxP3 expression

In normal healthy donors, $CD25^+TNFR2^+$ cells represented 5~12% of freshly isolated PB CD4 population (Fig 2A), which is substantially higher than the previously reported 1~2% $CD25^{high}$ Tregs (6) or <1% $HLA\text{-}DR^+$ Tregs (10), or 5.8% $CD25+CD127^{lo/-}$ Tregs (9). The proportion of human circulating $CD4^+CD25^+TNFR2^+$ cells is comparable to the reported 5~10% of $CD4^+CD25^+$ Tregs present in mouse peripheral lymphoid tissues (16, 19, 20). All human $CD25^{hi}$ cells were $TNFR2^+$ and therefore were included in the $CD25^+TNFR2^+$ subpopulation of CD4 cells (Fig 2A).

Since both $CD4^+CD25^{high}$ cells and $CD25^{low}FoxP3^+$ cells expressed high levels of TNFR2, we hypothesized that co-expression of TNFR2 and CD25 might identify more $FoxP3^+$ Tregs. As shown in Figure 2B~C, analysis of multiple donors revealed that greater than 85.3% on average (range: 72.8~94.1%) of $CD4^+CD25^+TNFR2^+$ cells expressed FoxP3. This is comparable to FoxP3 profile reported for human PB $CD4^+CD25^{high}$ cells (79.4~95.5%) (8) and $CD4^+CD25^+CD127^{low/-}$ Tregs (67.4~93.6%) (9). Consequently, the $CD4^+CD25^{high/low}TNFR2^+$ subset included a greater number of cells with FoxP3-expressing Treg phenotype than $CD4^+CD25^{high}$ cells. In contrast, $CD4^+CD25^+TNFR2^-$ cells, uniformly expressed low levels of CD25, only comprised 33.1% on average of FoxP3+ cells (range:

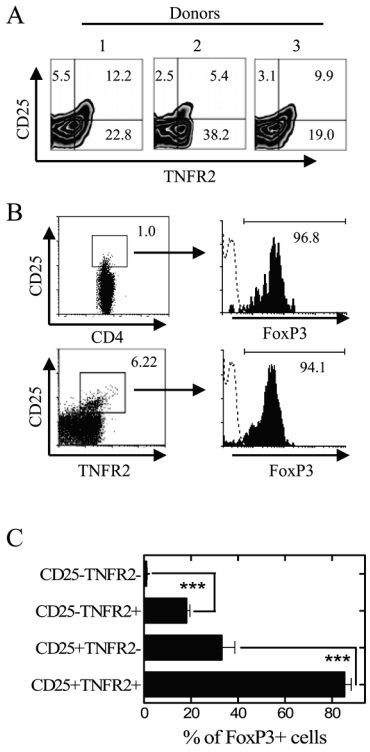

Figure 2. FoxP3 expression by human PB $CD4^+CD25^+TNFR2^+$ cells. Freshly isolated human PBMCs were stained for CD4, CD25 and TNFR2. The cells then were fixed and stained intracellularly for FoxP3. For FACS analysis, the PBMCs were gated on $CD4^+$ lymphocytes based on forward and side light scatter and CD4 staining. (A) Expression of CD25 and TNFR2 on PB CD4 cells. Data from three different donors are shown. The numbers in the quadrants indicate the percentage of positive cells. (B) Expression of FoxP3 by $CD4^+CD25^{hi}$ cells (upper panel) and $CD4^+CD25^+TNFR2^+$ cells (lower panel). Data shown are representatives of at least three separate experiments on different donors with similar results. The numbers in the dot plots are the number of gated cells. The numbers in histograms are the percentage of positive cells (%). (C) Expression of FoxP3 by different subsets of PB CD4 cells. The data shown are summarized from eight different donors (mean ± SEM, N=8). Comparison of percentage of $FoxP3^+$ cells in $CD25^+TNFR2^+$ subset and $CD25^+TNFR2^-$ subset or $CD25^-TNFR2^+$ subset and $CD25^-TNFR2^-$ subset: ** P<0.001.

20.0~54.8%), which was markedly lower than the 85.3% FoxP3-expressing cells present the $CD25^+TNFR2^+$ cells (P<0.001). Furthermore, only 18.1% on average of $CD25^-TNFR2^+$ cells were $FoxP3^+$ (range: 15.1~22.1%), while ~1% of $CD25^-TNFR2^-$ cells expressed FoxP3 (P<0.001). The intensity of FoxP3 expression on a per cell basis by $CD25^+TNFR2^+$ cells was also higher, than $CD25^+TNFR2^-$ cells and by $CD25^-TNFR2^+$ cells (data not shown). Therefore, co-expression of CD25 and TNFR2 is able to identify CD4 Tregs expressing high levels of $FoxP3^+$.

Phenotype of human PB $CD4^+CD25^+TNFR2^+$ cells

Surface expression of antigens by Tregs allows characterization of their phenotype and may also provide insight into their mechanism of action. Mouse $CD4^+CD25^+TNFR2^+$ T cells represent a subset of Tregs that have a memory/activated phenotype (15). We therefore investigated whether human PB $CD4^+CD25^+TNFR2^+$ T cells had similar phenotypic characteristics as their mouse counterparts. This was determined by comparing the HLA-DR, CTLA-4, FoxP3, CD45RO, CD45RA, CCR4 and CCR7 levels of the various subpopulations. As shown in Figure 3, $CD4^+CD25^+TNFR2^+$ cells expressed the highest

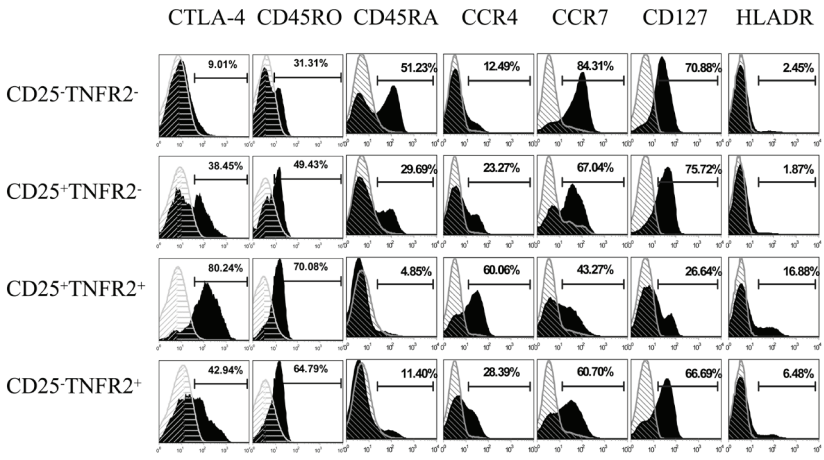

Figure 3. Phenotype of human PB CD4 subsets. Freshly isolated human PBMCs were stained for CD4, CD25, TNFR2 and additional phenotypic markers. Intracellular expression of CTLA-4 and surface expression of CD45RO, CD45RA, CCR4, CCR7, CD127 and HLA-DR by different subsets of PB CD4 cells, gating on indicated populations were analyzed by FACS. Black filled histogram: antibody staining; grey histogram: isotype control. Numbers in the figures indicate the percentage of gated cells expressing relevant marker. Data shown are representative of at least three separate experiments on different donors with similar results.

level of CD45RO (70.08%), a marker that is associated with proliferative responses to recall antigens, but the lowest level of CD45RA (4.85%), a marker of naïve CD4 cells, similar to the low level expression of CD45RB by mouse TNFR2$^+$ Tregs (15), indicative of a memory/activated phenotype. CTLA-4 is expressed predominantly by the Treg compartment of resting mouse CD4 cells and is critical for Treg function (21, 22). Human CD4$^+$CD25$^+$TNFR2$^+$ cells expressed markedly higher intracellular levels of CTLA-4 (80.24%) than CD4$^+$CD25$^+$TNFR2$^-$ cells (38.45%), CD4$^+$CD25$^-$TNFR2$^+$ cells (42.94%) or CD4$^+$CD25$^-$TNFR2$^-$ cells (9.01%). It has been shown that a CCL22-CCR4 signal was responsible for the trafficking of Tregs to human ovarian tumor (23). Of the CD4$^+$ subsets, CD4$^+$CD25$^+$TNFR2$^+$ cells expressed the highest level of CCR4 (60.06%), while other subsets only contained 12.49~28.39% of CCR4$^+$ cells. In contrast, human CD4$^+$CD25$^+$TNFR2$^+$ cells expressed a relatively low level of CCR7 (43.27%), a chemokine receptor responsible for directing traffic of naïve CD4 cells to the lymph nodes, as compared with 60~84% CCR7$^+$ cells in the other 3 subsets. As expected, CD4$^+$CD25$^+$TNFR2$^+$ cells were low in expression of CD127(9, 13), indicative of an inverse correlation of TNFR2 and CD127 as markers of Tregs. MHC-DR$^+$ Tregs (10) were largely

confined to the $CD4^+CD25^+TNFR2^+$ subset of cells. Thus, in addition to their high level of FoxP3 expression, human $CD4^+CD25^+TNFR2^+$ cells exhibited the phenotypic characteristics of functional Tregs.

Human PB $CD4^+CD25^+TNFR2^+$ cells are hyporesponsive to TCR stimulation and suppress responder T cells

The in vitro functional hallmarks of Tregs are that they are anergic to TCR stimulation and markedly suppress activation of co-cultured responder T cells. We therefore compared the response to TCR stimulation of $CD4^+CD25^+TNFR2^+$ and $CD4^+CD25^-TNFR2^-$ cells. TCR stimulation of $CD4^+CD25^-TNFR2^-$ Teffs resulted in robust proliferation and INFγ production. In contrast, $CD4^+CD25^+TNFR2^+$ cells were hyporesponsive, both in term of proliferation (Fig 4A) and INFγ production (Fig 4B), to TCR stimulation. Both $CD4^+CD25^+TNFR2^-$ cells and $CD4^+CD25^-TNFR2^+$ cells were more responsive than $CD4^+CD25^+TNFR2^+$ cells, but less responsive than $CD4^+CD25^-TNFR2^-$ cells, to TCR stimulation (data not shown).

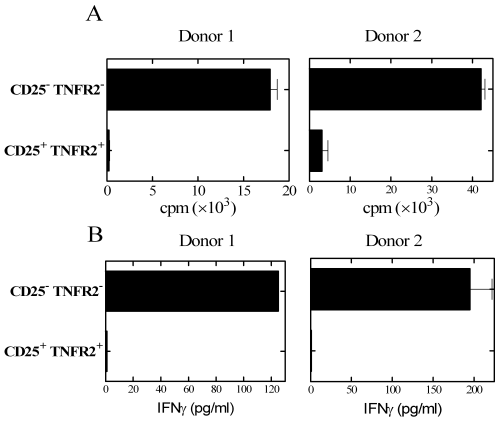

Figure 4. Comparison of functional capacities of human PB CD4 T cell subsets. $CD4^+CD25^+TNFR2^+$ and $CD4^+CD25^-TNFR2^-$ T cells were FACS-sorted from freshly isolated PBMCs. 2.5×10^4 cells/well of different CD4 subsets were cultured alone. The cells were stimulated with APCs and anti CD3 Ab for 72 h. Proliferation was determined by [^{3}H] thymidine incorporation assay (A). IFNγ level in the supernatants were determined (B). Data shown are two separate experiments on different donors.

Next, we examined the ability of $CD4^+CD25^+TNFR2^+$ T cells to suppress the proliferation of co-cultured responder Teffs. As shown in Figure 5A, $CD4^+CD25^+TNFR2^+$ T cells potently inhibited proliferation of co-cultured Teffs (Fig 5A) in a cell number dependent manner (Fig 5B), while $CD4^+CD25^+TNFR2^-$ and $CD4^+CD25^-TNFR2^+$ cells usually did not show inhibitory activity. Since $CD4^+CD25^+TNFR2^-$ and $CD4^+CD25^-TNFR2^+$ cells were not anergic and proliferated in response to TCR stimulation (data not shown), the [^{3}H] thymidine incorporation assay might not have revealed their suppressive potential. We therefore performed CFSE dilution assay in which MACS-purified autologous CD4 cells

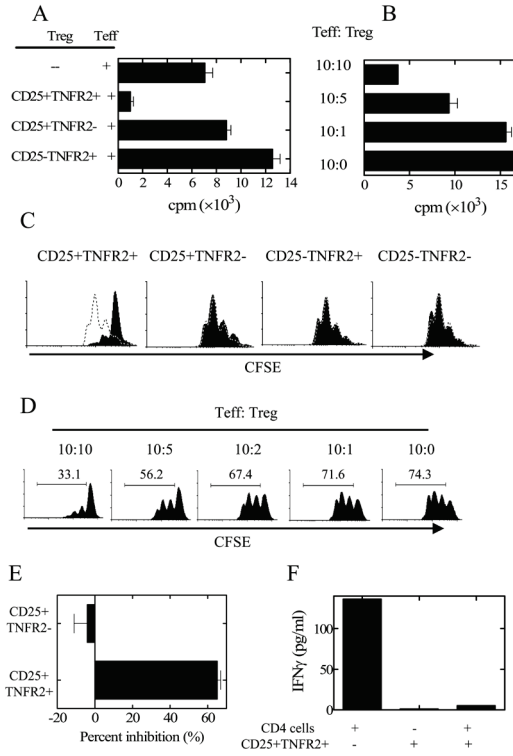

Figure 5. Suppressive activities of human PB CD4 T cell subsets. $CD4^+CD25^{+}TNFR2^+$, $CD4^+CD25^{+}TNFR2^-$, $CD4^+CD25^-TNFR2^+$ and $CD4^+CD25^-TNFR2^-$ T cells were FACS-sorted from freshly isolated PBMCs. In some experiment MACS-purified CD4 cells were used as responder cells. (A) 2.5×10^4 cells/well of $CD4^+CD25^-TNFR2^-$ cells, used as responder cells, were cultured alone or co-cultured with indicated CD4 subsets at ratio of 1:1. Background proliferation (APC alone, cpm: 1888.7) was subtracted. (B) 2.5×10^4 cells/well of $CD4^+CD25^-TNFR2^-$ cells were cultured alone or co-cultured with $CD4^+CD25^{+}TNFR2^+$ cells at indicated ratios. Proliferation was determined by [^{3}H] thymidine incorporation assay. (C) 5×10^4 cells/well of CD4 cells were labeled with CFSE, cultured alone or co-cultured with indicated CD4 subsets at 1:1 ratio. (D) 5×10^4 cells/well of MACS-purified CD4 cells were labeled with CFSE, cultured alone or co-cultured with indicated ratio with FACS-purified $CD4^+CD25^{+}TNFR2^+$ cells. After 72 h incubation, dilution of CFSE by responder CD4 cells was determined by FACS. The cultured cells were stimulated with APCs and anti CD3 Ab. (A~D) Representative data from 3 separate experiments on different donors with similar results were shown. (E) Percent inhibition of proliferation of $CD4^+CD25^-TNFR2^-$ T cells by $CD4^+CD25^{+}TNFR2^-$ cells and $CD4^+CD25^{+}TNFR2^+$ cells [(CPM (responder cells alone)-CPM (responder cells co-cultured with Treg cells))/CPM (responder cells alone)×100%]. Data shown were summarized from three separate experiments on different donors. (F) MACS-purified CD4 cells and FACS-purified $CD4^+CD25^{+}TNFR2^+$ cells were cultured alone or co-cultured at ratio of 1:1. The cells were stimulated with APCs and anti CD3 Ab for 72 h. IFNγ level in the supernatants were determined. Data shown are representative of three separate experiments on different donors with similar results. Data in (E-F) are presented as Mean and SEM.

were labeled with CFSE and co-cultured with FACS-purified CD4 subsets. $CD4^+CD25^+TNFR2^+$ T cells consistently markedly suppressed the replication of responder CD4 cells (Fig 5C) in a dose-dependent manner (Fig 5D). In contrast, the other three CD4 subsets, including $CD4^+CD25^+TNFR2^-$ and $CD4^+CD25^-TNFR2^+$ cells, did not show inhibitory activity. The anti-proliferative activity of $CD4^+CD25^+TNFR2^+$ cells was consistently seen with multiple donors (Fig 5E). Furthermore, $CD4^+CD25^+TNFR2^+$ T cells almost completely inhibited cytokine (INFγ) production by co-cultured Teffs (Fig 5F). Thus, co-expression of CD25 and TNFR2 on CD4 cells was able to identify functional suppressive human PB CD4 Tregs which were also anergic.

Discussion

Here we report that the phenotypic and functional characteristics of human $CD4^+CD25^+TNFR2^+$ Tregs present in normal donor peripheral blood closely resemble mouse $CD4^+CD25^+TNFR2^+$ Tregs. Virtually all human $CD4^+CD25^{high}FoxP3^+$ cells plus a substantial proportion of $CD4^+CD25^{low}FoxP3^+$ cells expressed TNFR2. Thus, the population of $CD4^+CD25^{high/low}TNFR2^+$ cells include 5-fold or more $FoxP3^+$ Tregs as compared with the number of Tregs identified by $CD25^{high}$. More than 90% of human PB $CD4^+CD25^+TNFR2^+$ cells were $FoxP3^+$, which is comparable to the FoxP3 profile of mouse $CD4^+CD25^+$ Tregs (~90%) (16, 19) and FoxP3 profile of reported human $CD4^+CD25^+CD127^{low/-}$ Tregs (86.6%) (9).

These PB $CD4^+CD25^+TNFR2^+$ cells co-expressed high levels of FoxP3 and also functioned as Tregs. As revealed in a standard in vitro Treg function assay, $CD4^+CD25^+TNFR2^+$ cells at a 1:1 ratio to $CD4^+CD25^-TNFR2^-$ cells reproducibly reduced proliferation by the co-cultured Teffs by greater than 60%. It was previously reported that human PB $CD4^+CD25^{high}$ Tregs reduced [^{3}H] thymidine incorporation of co-cultured $CD4^+CD25^-$ responder cells by 69% at day 5 and >98% by day 7 (6). Thus, the average inhibition of > 60% of $CD4^+CD25^+TNFR2^+$ cells on day 3 in our study is comparable to the level of inhibition exerted by $CD4^+CD25^{high}$ at day 5. We evaluated the suppressive effect on highly proliferative $CD4^+CD25^-TNFR2^-$ cells were used as responder cells. Other investigators have reported more profound suppression because they used less proliferative CD4 cells or $CD4^+CD25^-$ cells which still contained $CD4^+CD25^-TNFR2^+FoxP3^+$ cells as responder cells. Production of cytokines by Teffs is usually more susceptible to inhibition by Tregs than their proliferation. Indeed, at a 1:1 ratio of $CD4^+CD25^+TNFR2^+$ cells to CD4 responder cells, the production of INFγ by responder cells was almost completely inhibited. Consequently, the subset of human PB $CD4^+CD25^+TNFR2^+$ cells is profoundly suppressive.

It was recently reported that both human and mouse Tregs were able to shed large amounts of soluble TNFR2 (sTNFR2) upon stimulation with IL-2 and plate-bound anti-CD3, and consequently, the resultant sTNFR2 was proposed to provide a means by which Tregs inhibited the activation of Teffs (18). However, we did not detect an elevated level of soluble TNFR2 in the supernatant in our in vitro culture of human $CD4^+TNFR2^+$ cells stimulated with APCs and soluble anti CD3 for 72 hrs (data not shown), conditions commonly used to exhibit inhibitory activity of Tregs in vitro (24). Furthermore, FACS-purified mouse and human $CD4^+CD25^+TNFR2^+$ cells did not release TNFR2 into the supernatants under same conditions (data not shown). Although $CD4^+CD25^+TNFR2^+$ cells had potent suppressive capacity, another CD4 subset which also expressed TNFR2, e.g. $CD4^+CD25^-TNFR2^+$ cells, did not have any suppressive activity, mitigating against an inhibitory role for sTNFR2. Although we have not ruled out a role for sTNFR2 as a basis for suppressive effects of Tregs in vivo, shedding of sTNFR2 can not explain the potent suppression mediated by $CD4^+CD25^+TNFR2^+$ cells in the in vitro Treg functional assay.

Human PB $CD4^+CD25^+TNFR2^+$ cells, akin to their mouse counterpart, expressed strikingly high levels of CTLA-4 (Fig 3), as compared with the other CD4 subsets. CTLA-4 has been recently found to play a key role in the Treg suppressive effect in mice (22, 25). Whether CTLA-4 accounts for the suppressive activity of human PB $CD4^+CD25^+TNFR2^+$ Tregs will be addressed in a future study.

TNFR2 is one of two receptors for TNF, a pleiotropic cytokine which is a major participant in the initiation and orchestration of inflammation and immunity (26). Unlike TNFR1 which can mediate cytotoxic effect through its death domain, TNFR2 does not have a death domain and is largely confined to lymphoid cells (26). Several lines of evidence indicate that TNFR2 acts as a co-stimulator for antigen-driven T cell responses (26-29). The preferential expression of TNFR2 on mouse and human Tregs suggest that this receptor may mediate the activating effect of TNF on Tregs (16). Similar to our observation that TNF plus IL-2 selectively promotes the proliferation of mouse $FoxP3^+$ Tregs (16), human $FoxP3^+$ cells present in the CD4 population were also expanded in response to TNF stimulation in conjunction with IL-2 (data not shown), suggestive of a functional role of TNFR2 in the expansion of human Tregs. Presumably, high levels of TNFR2 expression by human Tregs may mediate a quick response to inflammation followed by a negative feedback attenuation of inflammatory responses that serves to prevent collateral self-tissue damage. Thus, IL-2 is essential for the homeostatic survival and proliferation of Tregs, whereas TNF results in a proliferative expansion of Tregs in an inflammatory environment

(15, 16). A recent report showing that expansion of TNFR2+ Tregs with enhanced suppressive activity occurs in malaria patients may reflect this scenario (30).

One of the difficulties in studies of human Tregs is to identify active Tregs present in the subset of $CD25^{int/low/-}$ CD4 cells with suppressive biological function. For example, it was recently reported that $CD45RA^{+}FoxP3^{+}$ "naive Tregs", which have the capacity to be expanded in vitro into homogenous functional Tregs (31), were $CD25^{int}$ cells (32). Our data showed that FoxP3-expressing cells, including those present in $CD25^{-}$ population, expressed high levels of TNFR2 (Fig 1). To date, it is impossible to study the function of $CD25^{-}FoxP3^{+}$ cells, since their specific surface markers for viable isolation are not clear. How to exploit TNFR2 expression to identify these $CD25^{-}FoxP3^{+}$ cells therefore merit more study.

Taken together, our data demonstrate that the combination of CD4, CD25 and TNFR2 identifies a larger population of functional Tregs in the circulation of normal humans. This population of T cells was practically identical to mouse $CD4^{+}$ Tregs, in manifesting high level expression of FoxP3 and CTLA-4, hypo-responsiveness to TCR cross-linking and potent inhibition of activation of co-cultured T responder cells. Identification of human Tregs with CD4, CD25 and TNFR2 provides over 5-fold better detection and viable enrichment of human Tregs than commonly used $CD25^{high}$ method for in vitro studies and potentially for in vivo therapy.

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Chapter 5

TNF by inducing co-stimulatory TNF receptor superfamily members TNFR2, 4-1BB and OX40 augments the number and function of mouse $CD4^+FoxP3^+$ regulatory T cells

Ryoko Hamano, Jiaqiang Huang, Teizo Yoshimura, Joost J. Oppenheim and Xin Chen

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Abstract

TNF is a pleiotropic cytokine with intriguing biphasic pro-inflammatory and anti-inflammatory effects. Our previous studies demonstrated that TNF up-regulated FoxP3 expression and activated and expanded $CD4^+FoxP3^+$ regulatory T cells (Tregs) by utilizing TNFR2. Further, TNFR2-expressing Tregs exhibited maximal suppressive activity. In this study, we show that TNF, in concert with IL-2, preferentially up-regulated mRNA and surface expression of TNFR2, 4-1BB and OX40 on Tregs. Agonistic antibodies against 4-1BB and OX40 also induced the proliferation of suppressive Tregs. Thus, TNF amplifies its stimulatory effect on Tregs by inducing TNF receptor superfamily (TNFRSF) members. In addition, administration of neutralizing anti-TNF Ab blocked LPS-induced expansion of splenic Tregs and up-regulation of TNFR2, OX40 and 4-1BB receptors on Tregs in vivo, indicating that the expansion of Tregs expressing these co-stimulatory TNFRSF members in response to LPS is mediated by TNF. Taken together, our novel data indicate that TNF preferentially up-regulates TNFR2 on Tregs, and this is amplified by the stimulation of 4-1BB and OX40, resulting in the optimal activation of Tregs and augmented attenuation of excessive inflammatory responses.

Introduction

$CD4^+FoxP3^+$ regulatory T cells (Tregs) comprise only a minor fraction (~10%) of peripheral CD4 cells, but play a critical role in the establishment and maintenance of immunological tolerance to self antigens as well as to foreign antigens (1, 2). Certain cytokine receptors preferentially expressed by Tregs not only serve as surface markers for the identification of Tregs, but also promote the function of Tregs. CD25, the α chain of IL-2 receptor, is the prototype of such cytokine receptors (1, 2). Our previous studies indicate that TNFR2 is an important cytokine receptor preferentially expressed by the highly suppressive human and mouse Tregs (3-5).

TNFR2 is one of two receptors transducing the biological function of TNF, a pleiotropic cytokine which is a major participant in the initiation and orchestration of inflammation and immunity (6). TNFR2 expression is restricted to certain T cell subpopulations (6), and acts as a co-stimulator for antigen-driven T cell responses (7). We have found that TNF surprisingly was an activator of Tregs, resulting in their proliferative expansion, up-regulation of FoxP3 expression and increase of suppressive activity (3). This data, albeit counterintuitive, is supported by the emerging evidence that TNF-TNFR2 interaction plays a critical role in the generation, expansion and function of human and mouse Tregs (8-12).

TNFR2 is constitutively expressed by human and mouse thymic Tregs (5, 13). Normal human circulating Tregs expressed markedly higher levels of TNFR2 than $CD4^+FoxP3^-$ effector T cells (Teffs) (4, 14, 15). Normally 30~40% of Tregs present in the peripheral lymphoid tissues of unstimulated Balb/c mice and C57BL/6 (B6) mice expressed a high level of TNFR2, while less than 10% of Teffs expressed a lower level of TNFR2 (3, 16). Furthermore, TNFR2-expresssing Tregs exhibited the most potent suppressive activity, while $TNFR2^-$ Tregs even though $CD25^+$ and $FoxP3^+$ in normal C57BL/6 mice had only minimal or no suppressive activity (5, 16). Intratumoral Tregs are maximally immunosuppressive, since the majority of tumor infiltrating Tregs were highly suppressive $TNFR2^+$ cells (5, 16), and depletion of $TNFR2^+$ Tregs was associated with tumor eradication after cyclophosphamide treatment (17). When transferred to LPS-challenged recipient mice, Tregs from wild-type mice were able to inhibit inflammatory responses, while Tregs from TNFR2-deficient mice failed to do so (14). In normal human peripheral blood (PB), TNFR2-expressing $CD4^+CD25^+$ cells comprised a high level of $FoxP3^+$ cells

and were functionally suppressive (4). In malaria patients, proliferating $TNFR2^+$ Tregs exhibited an enhanced suppressive activity (18). These studies clearly demonstrate that TNFR2 not only serves as a marker, but also promotes Treg function.

We have investigated the effect of TNF on TNFR2 expression on Tregs. Since TNFR2 is a member of TNF receptor superfamily (TNFRSF) and other co-stimulatory TNFRSF members, such as 4-1BB (19) and OX40 (20) also have been reported to participate in Treg activity, we also investigated their response to TNF. We found that TNF preferentially up-regulates these TNFRSF on Tregs, which contribute to the optimal activation of Tregs and result in attenuation of excessive inflammatory responses.

Materials and Method

Mice and reagents. Female wild type (wt) C57BL/6 mice were provided by the Animal Production Area of the National Cancer Institute (Frederick, MD). NCI-Frederick is accredited by American Association for the Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the Procedures outlined in the "Guide for Care and Use of Laboratory Animals" published by the National Research Council (National Research Council, National Academy of Sciences. (1996) Guide for Care and Use of Laboratory Animals. National Academy Press, Washington D.C. FoxP3/gfp KI mice were kindly provided by Dr. Yasmine Belkaid at Laboratory of Parasitic Diseases, NIAID, NIH, and maintained in the Animal Production Area of the NCI-Frederick.

Antibodies purchased from BD Pharmingen (San Diego, CA) consisted of PerCP anti-mouse CD3 (145-2C11), PE and APC and Pacific blue anti-mouse CD4 (RM4-5), FITC anti-mouse CD44 (IM7), PE anti-mouse CD120b/TNFR2 (TR75-89) and FITC anti-mouse CD90/FAS (Jo2). FITC and PerCP Cy5.5 anti-mouse CD4 (L3T4), FITC anti-mouse CD69 (H1.2F3), FITC anti-mouse GITR (DTA-1), PE anti-mouse CD134/OX40 (OX-86), PE anti-mouse CD137/4-1BB (17B5), PE and APC and eFuor 450 anti-mouse/rat FoxP3 staining set (FJK-16s), and functional grade purified anti-mouse IL-2 (JES6-1A12), CD137 (17B5) and CD134 (OX-86) were purchased from eBioscience (San Diego, CA). LEAF™ purified anti-mouse CD252 (OX40 ligand, RM134L) and LEAF™ purified anti-mouse CD137 ligand (4-1BB ligand, TKS-1) was purchased from Biolegend (San Diego, CA). Alexa 647 anti-mouse CD120b/TNFR2 (TR75-89) was purchased from Serotec (Raleigh, NC). Murine IL-2, IL-7 and TNF were purchased from PeproTech (Rocky Hill, NJ). A neutralizing anti-mouse TNF Ab (5E5) and murine IgG1 were generously provided by Drs. Teresa Born and John E. Sims (Amgen Inc., Seattle, WA).

Cell purification. Mouse lymphocytes were harvested from mouse spleens, axillary lymph nodes, inguinal lymph nodes and mesenteric lymph nodes. $CD4^+$ cells were purified from lymphocytes with mouse CD4 (L3T4) MicroBeads and LS column (Miltenyi Biotec, Auburn, CA). CD4 subsets were purified using Cytomation MoFlo cytometer (Fort Collins, CO), yielding a purity of ~98% for each subsets. T-depleted spleen cells were used as APCs and were prepared by depletion of $CD90^+$ cells with anti-mouse CD90 MicroBeads and LD column (Miltenyi Biotec). APCs were irradiated with 3,000 R.

In vitro cell culture and Treg function assay. In order to examine surface expression of TNFRSFs, $CD4^+$ cells were cultured at 10^5 cells/well in a 96-well plate with medium (3) alone or IL-2 or IL-7 with or without TNF, or with neutralizing anti-IL-2 Ab, for desired time. Unless otherwise specified, the concentration of cytokines used in vitro cultures was 10 ng/ml, and the concentration of antibodies was 10 μg/ml. The surface expression of TNFRSFs and other markers on Tregs or Teffs was analyzed with FACS, by gating on $FoxP3^+$ or $FoxP3^-$ cells. In some experiments, flow-sorted $CD4^+FoxP3/gfp^+TNFR2^-$ cells or $CD4^+FoxP3/gfp^-TNFR2^-$ cells from FoxP3/gfp KI mouse spleen and LNs were treated with IL-2 or IL-2 plus TNF. After 72-hr incubation, surface expression of TNFR2 was determined with FACS.

In some experiments, Flow-sorted $CD4^+FoxP3/gfp^+$ Tregs ($2\sim5\times10^4$ cells/well) were cultured in a U-bottom 96-well plate with IL-7 or with IL-2, with or without TNF, or with agonistic Abs for OX40 or 4-1BB, or with antagonistic Abs for OX40 L or 4-1BB L. The cells were stimulated with 2×10^5 APCs/well plus 0.5 μg/ml of soluble anti-CD3 Ab. Cells were pulsed with 1 μCi [3H]thymidine (Perkin Elmer Life Sciences, Boston, MA) per well for the last 6 hr of the culture period.

In order to determine Treg function, CFSE-labelled responder Teffs (5×10^4 cells/well) were seeded in a U-bottom 96-well plate together with 2×10^5 cells/well of APCs and 0.5 μg/ml of anti-CD3 antibody. Flow-purified $CD4^+CD25^+$ cells were added to the wells at the desired ratio. After 48 hours, CFSE dilution was determined with FACS. In some experiments, flow-sorted Tregs were treated with TNF/IL-2, with or without agonistic anti-4-1BB Ab or agonist anti-OX40 Ab, for 72 hours. After thoroughly washing, pretreated Tregs were co-cultured with freshly isolated Teffs at the desired ratio to observe their suppressive potential.

In vivo administration of LPS and anti-mouse TNF Ab. Normal C57BL/6 mice were injected intraperitoneally with 200 μg of LPS (Sigma-Aldrich, St. Louis, MO, Cat#: L9764) in PBS. In some experiments, mice were injected (i.p.) with 200 μg of a neutralizing anti-

mouse TNF Ab (5E5) or Mu IgG1 24 hours and 1 hour before injection of LPS. Mouse spleens and mesenteric LNs were harvested at 0, 6, 24, 48 and 72 hours after injection for the FACS analysis of phenotype.

Quantitative real time RT-PCR assay analysis of Tnfrsf genes. RNA samples were extracted from flow-sorted $CD4^+FoxP3/gfp^+$ or $CD4^+FoxP3/gfp^-$ cells as described and reverse transcribed. Quantitative real time PCR was performed to determine relative mRNA expression using primers specific to Tnfrsf genes (SABiosciences RT² qPCR Primer Assays). Ct values from each gene in each sample were normalized using the $2^{-\Delta\Delta Ct}$ calculation method with Gapdh gene as housekeeping gene by following SABiosciences' instruction.

Flow Cytometry. After blocking FcR, cells were incubated with appropriately diluted antibodies. Acquisition was performed using a FACSort or a LSRII (BD Biosciences, Mountain View, CA) and data analysis was conducted using FlowJo software (Tree Star Inc., Ashland, OR).

Statistical analysis. Comparisons of two groups of data were analyzed by two-tailed Student's t test using GraphPad Prism 4.0. (GraphPad, San Diego, CA)

Results

TNF, in the presence of IL-2, induces Tregs to express genes encoding costimulatory TNFRSF members

In order to test the effect of TNF on the expression of TNFR2 and other co-stimulatory TNFRSF members on Tregs, we performed gene profiling assay using the Mouse Tumor Necrosis Factor (TNF) Ligand and Receptor Signaling Pathways RT² Profiler™ PCR Array (SABiosciences, Frederick, MD). This showed that, by comparison with freshly isolated Tregs or with TNF/IL-2-treated Teffs, Tregs treated with TNF/IL-2 for 12 hrs up-regulated their expression of genes encoding a number of TNFRSF members, including Tnfrsf1b (TNFR2), Tnfrsf4 (OX40), Tnfrsf6 (FAS), Tnfrsf9 (4-1BB), and Tnfrsf18 (GITR), by greater than 2-fold (data not shown). Our results are in agreement with a recent microarray study in human Tregs (15). We next performed real time PCR assay to verify their changes in gene expression. As shown in Fig 1A, the expression of mRNA for TNFR2, OX40, 4-1BB and GITR was two-fold higher in freshly isolated Tregs than freshly isolated Teffs (left). After treatment with TNF/IL-2, the expression of mRNA for these TNFRSF members and FAS was at least two-fold higher in Tregs than in Teffs (middle). Treatment with TNF/IL-2 further up-regulated their mRNA expressions greater than 4-fold in Tregs,

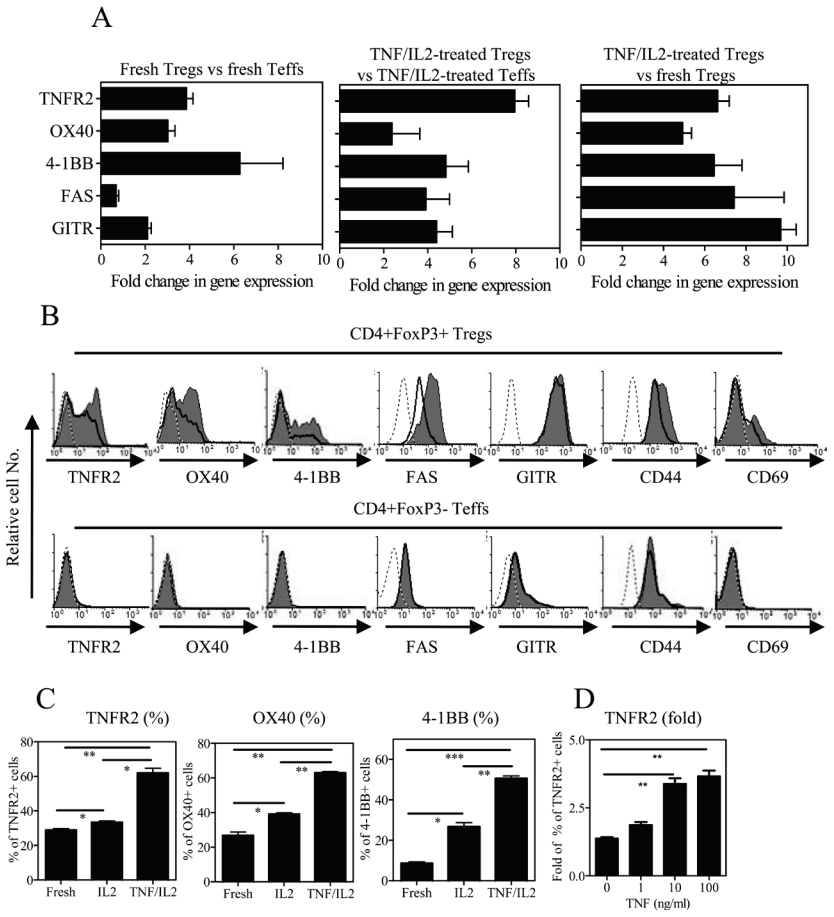

Figure 1. Up-regulation of genes and surface expression of co-stimulatory TNFRSF members on Tregs by TNF. (A) Flow-sorted $CD4^+FoxP3/gfp^+$ Tregs and $CD4^+FoxP3/gfp^-$ Teffs were cultured with TNF and IL-2 for 12 hours. RNA from freshly purified cells and TNF/IL-2-treated cells was isolated and gene expression of TNFRSF members was analyzed by real time PCR. Fold changes in gene expression between two indicated groups are shown. The data (means ± SEM, n=4) are summarized from four separate experiments with similar results. (B-D) CD4 cells were incubated with IL-2 or TNF/TNF for 3 days. The surface markers were analyzed with FACS by gating on $FoxP3^+$ Tregs or $FoxP3^-$ Teffs. (B) Typical results of FACS analysis. Grey: TNF/IL-2; solid line: IL-2; dashed line: isotype control. (C) Summary of the percentage of $TNFR2^+$, $OX40^+$, and $4\text{-}1BB^+$ cells in Tregs. (D) In the presence of consistent concentration of IL-2, TNF up-regulated TNFR2 expression on $FoxP3^+$ Tregs in a dose-dependent manner. Data shown are fold change in percentage of $TNFR2^+$ Tregs over that of freshly isolated Tregs. Comparison of two indicated groups: *p<0.05, ** p<0.01, *** p<0.001. Data shown in C-D (means ± SEM, n=3) are representative of at least three separate experiments with similar results.

as compared with freshly isolated Tregs (right). Thus, in the presence of IL-2, TNF up-regulated the gene expression of TNFR2 and other co-stimulatory TNFRSF members in Tregs.

TNF, in concert with IL-2, up-regulates surface expression of co-stimulatory TNFRSF members on Tregs

Treatment with TNF/IL-2 for three days preferentially up-regulated the surface expression of TNFR2, OX40, 4-1BB and FAS on Tregs, but not on Teffs (Fig 1B). TNFR2, OX40 and 4-1BB expressed on IL-2/TNF-treated Tregs were increased by 2.1±0.2, 2.4±0.2 and 6.0±0.7 fold, respectively, over their expression on freshly isolated Tregs ($p<0.05$~0.001, Fig 1C). IL-2 alone also increased their surface expression ($p<0.05$), however, addition of TNF further increased their expression by up to ~2-fold over IL-2 alone ($p<0.05$~0.01, Fig 1C). TNF-induced up-regulation in the case of TNFR2 was dose-dependent (Fig 1D). TNF was also able to up-regulate surface expression of TNFR2, OX40 and 4-1BB on FACS-purified $CD4^+FoxP3/gfp^+$ Tregs (data not shown), indicating that TNF directly acts on Tregs. The increased expression of these co-stimulatory TNFRSF members has been reported to be a consequence of the activation of CD4 cells (21). Indeed, IL-2/TNF treatment markedly and preferentially enhanced the expression of the activation markers, CD44 and CD69, on Tregs (Fig 1B). Therefore, IL-2/TNF led to greater activation of Tregs.

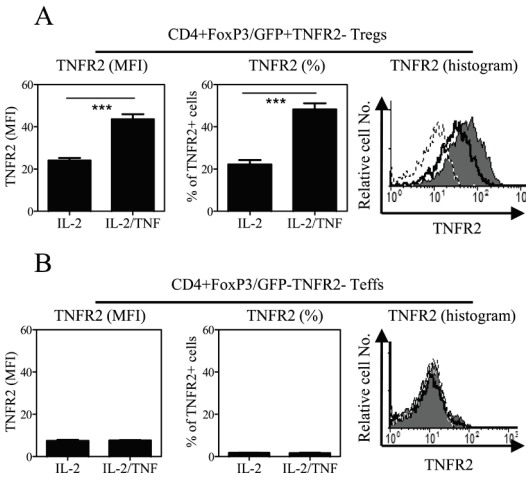

Figure 2. Induction of the expression of TNFR2 by IL-2 and TNF/IL-2 on $FoxP3^+TNFR2^-$ Tregs. $CD4^+FoxP3/gfp^+TNFR2^-$ cells and $CD4^+FoxP3/gfp^-TNFR2^-$ cells were flow-sorted. The cells were cultured with IL-2 or TNF/IL-2 for 3 days. The surface expression of TNFR2 was determined with FACS. Data (means ± SEM, n=3) in the left panel show the MFI of TNFR2 expression, and in the middle panel show the percentage of $TNFR2^+$ cells. The comparison of IL-2 and TNF/IL-2: *** $p<0.001$. Data in the right panel show results of a representative FACS analysis. Grey: TNF/IL-2; solid line: IL-2 alone; dashed line: isotype control. Data shown are representative of three separate experiments with similar results.

TNF/IL-2 induces TNFR2 expression on $TNFR2^-$ Tregs

It is possible that TNF, in addition to expanding $TNFR2^+$ Tregs, also converts

TNFR2$^-$ Tregs into TNFR2$^+$ Tregs. To test this, flow-sorted CD4$^+$FoxP3/gfp$^+$TNFR2$^-$ cells and CD4$^+$FoxP3/gfp$^-$TNFR2$^-$ cells were treated with IL-2 or TNF/IL-2. As shown in Fig 2A, IL-2 alone induced the expression of TNFR2 on FoxP3/gfp$^+$TNFR2$^-$ Tregs. Presumably based on the initial induction of TNFR2 by IL-2, TNF further amplifies the expression levels of TNFR2 on FoxP3/gfp$^+$TNFR2$^-$ Tregs ($p<0.001$). In contrast, neither IL-2 nor TNF/IL-2 was able to induce TNFR2 expression on FoxP3/gfp$^-$TNFR2$^-$ Teffs (Fig 2B). Thus, TNF does have the capacity to induce nonfunctional TNFR2$^-$ Tregs into functional TNFR2$^+$ Tregs.

IL-2-independent effect of TNF on the up-regulation of FoxP3 and TNFR2 expression by Tregs

Treatment with TNF/IL-2 was previously shown to up-regulate the expression of CD25 on Tregs(3). Thus the activating effects of TNF/IL-2 on Tregs and their stimulation of TNFR2 expression may depend entirely on the enhanced interaction of IL-2 with CD25. To test this hypothesis, we examined the effect of the combination of TNF and IL-7, another cytokine that uses common gamma chain and maintains the survival of Tregs in vitro (22). Only 6% of Tregs, and approximately same proportion of Teffs, were induced to proliferate when CD4 cells were cultured with IL-7 alone (Fig 3A left panels). In the presence of IL-7, TNF stimulation resulted in 37.8% replicating cells in the FoxP3$^+$ subset. In contrast, TNF treatment resulted in replication of only 10.8% of FoxP3$^-$ cells (Fig 3A right panels). Thus, IL-7 also enabled TNF to preferentially stimulate the proliferation of Tregs ($p<0.001$, Fig 3B). We also investigated the effect of IL-7 with or without TNF on the proliferative responses of flow-sorted CD4$^+$FoxP3/gfp$^+$ Tregs to TCR stimulation. As shown in Fig 3C, although IL-7 by itself only had minimal effect, a combination of TNF and IL-7 synergistically promoted the proliferation of Tregs.

Next, we examined the effects of TNF/IL-7 on the expression of FoxP3 and TNFR2 on Tregs. As shown in Fig 3D, after 3-day treatment with IL-7 alone, the proportion of FoxP3$^+$ Tregs present in CD4 cells was only ~4%, which was lower than that in freshly isolated CD4 cells (~10%) or CD4 cells cultured with IL-2 (10 ng/ml, >10%) for 3 days. Even at the higher molar concentration of IL-7 was not as effective as IL-2 in the maintenance of survival of Tregs. Nevertheless, TNF in conjunction with IL-7 was able to increase the proportion of FoxP3$^+$ cells (Fig 3D), in a dose-dependent manner (Fig 3E). Furthermore, in the presence of IL-7, TNF increased the proportion of TNFR2$^+$ cells in the FoxP3$^+$ subset,

but not in FoxP3$^-$ cells (Fig 3F), indicating that IL-7 could also promulgate the Treg-activating effect of TNF. In order to eliminate a possible effect of IL-2 released by

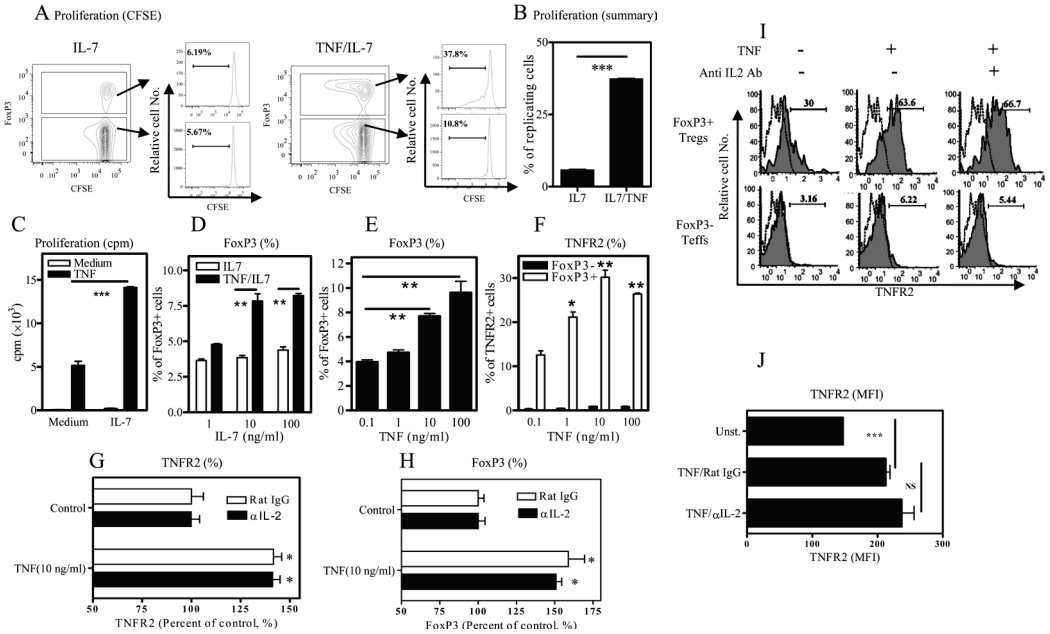

Figure 3. Stimulation of Tregs in the presence of IL-7 by TNF. (A-B) CD4 cells were labeled with CFSE and cultured with IL-7, in the absence or presence of TNF. After 3 days, the cell replication, as shown by dilution of CFSE, on Tregs and Teffs was analyzed with FACS. (C) Flow-sorted $CD4^+FoxP3/gfp^+$ T cells were stimulated with APCs and anti-CD3, in the presence of medium, or IL-7, or TNF, or TNF/IL-7. After 72-hour incubation, cell proliferation was determined by [3]H thymidine incorporation assay. (D-E) CD4 cells were cultured with increasing concentrations of IL-7 alone or with consistent concentration of TNF (D), or cultured with consistent concentration of IL-7 and increasing concentrations of TNF (E), for 3 days. The proportion of $FoxP3^+$ cells was analyzed by FACS. (B-E): ** $p<0.01$, *** $p<0.0001$, as compared with indicated group. (F) CD4 cells were cultured same as (D). The proportion of $TNFR2^+$ cells was analyzed with FACS by gating on $FoxP3^+$ cells or $FoxP3^-$ cells. *$p<0.05$; ** $p<0.01$, as compared with lowest dose of TNF (0.1 ng/ml). (G-H) CD4 cells were cultured with IL-7 plus 0.1 ng/ml of TNF as control or 10 ng/ml of TNF, with Rat IgG or neutralizing anti-IL-2 Ab. The proportion of TNFR2 (G) or FoxP3 (H) expression on Tregs was analyzed with FACS. Data shown are percentage of control (%). Comparison with respective control, * $p<0.05$. (I-J) CD4 cells were cultured with TNF alone, with or without neutralizing anti-IL-2 Ab, for 24 hours. The expression of TNFR2 was analyzed with FACS, by gating on $FoxP3^+$ cells or $FoxP3^-$ cells. Comparison of indicated two groups, *** $p<0.001$. NS: no statistical significance. Data in (B-H, J) are means ± SEM (N=3). Data shown are representatives of at least three separate experiments with similar results.

activated FoxP3- Teffs present in the unfractionated CD4 cells, neutralizing anti-IL-2 Ab was used. As shown in Fig 3G-H, in the presence of as high as 10 μg/ml of neutralizing anti-IL-2 Ab, TNF/IL-7 still up-regulated TNFR2 expression on Tregs and expanded $FoxP3^+$ cells ($p<0.05$). Furthermore, treatment with TNF alone for 24 hrs also resulted in an increase of TNFR2 expression on Tregs, which was not blocked by the neutralizing anti-IL-2 Ab (Fig 3I-J). Thus, the effect of TNF on the proliferation of Tregs and up-regulation of TNFR2 on Tregs can occur independently of IL-2.

Effects of Activation of 4-1BB and OX40 on Tregs

Next we examined whether 4-1BB and OX40 induced on Tregs by TNF were functional. As shown in Fig 4A-B, both agonistic anti-4-1BB and anti-OX40 Abs were able to partially overcome the anergic status of Tregs and induced proliferation of Tregs. Furthermore, the combination of TNF and anti-4-1BB Ab or anti-OX40 Ab synergistically stimulated the proliferation of Tregs ($p<0.05$~0.001. Fig 4A-B). In contrast, isotype control IgGs did not have any effect (data not shown). CD4-depleted splenocytes were used as APCs in this study and they expressed OX40 L and 4-1BB L (data not shown). We thereforeexamined the effect of blockade of OX40 L and 4-1BB L on the proliferation of Tregs. As shown in Fig 4C, TNF-induced the proliferative responses of $CD4^+FoxP3/gfp^+$ Tregs to APCs stimulation was partially abrogated by blocking antibodies to OX40 L and to a greater extent by anti-4-1BB L Ab ($p<0.05$). Thus, TNF-induced up-regulation of 4-1BB and OX40 appeared to augment the stimulatory signaling of agonistic Abs or their cognate ligands. In order to examine the effect of OX40 and 4-1BB activation on FoxP3 expression, $CD4^+FoxP3/gfp^+$ Treg cells were cultured in vitro with IL-2, or TNF/IL-2 with or without agonistic Abs for OX40 or 4-1BB. After 3-day culture, the levels of FoxP3 expression on a per cell basis (MFI) on Tregs was increased by ~2-fold after TNF/IL-2 treatment, as compared with IL-2 treatment alone ($p<0.001$, Fig 4D). Importantly, the TNF/IL-2-induced enhancement of FoxP3 expression in Tregs was preserved and even modestly increased by treatment with the 4-1BB agonistic Ab ($p<0.05$, Fig 4D). However, in our experimental system, the agonistic Abs for OX40 and 4-1BB did not further enhance TNFR2 expression on Tregs (data not shown), suggesting that the effect of TNF on the up-regulation of co-stimulatory TNFRSFs was unidirectional.

Next, the suppressive capability of Tregs expanded by the combination of TNF and anti-4-1BB Ab or anti-OX40 Ab was investigated. Consistent with our previous report (3), the

suppressive activity of Tregs pre-treated with TNF/IL-2 on the proliferation by Teffs was markedly enhanced (Fig 4E). Moreover, Tregs pre-treated with TNF/IL-2 in combination

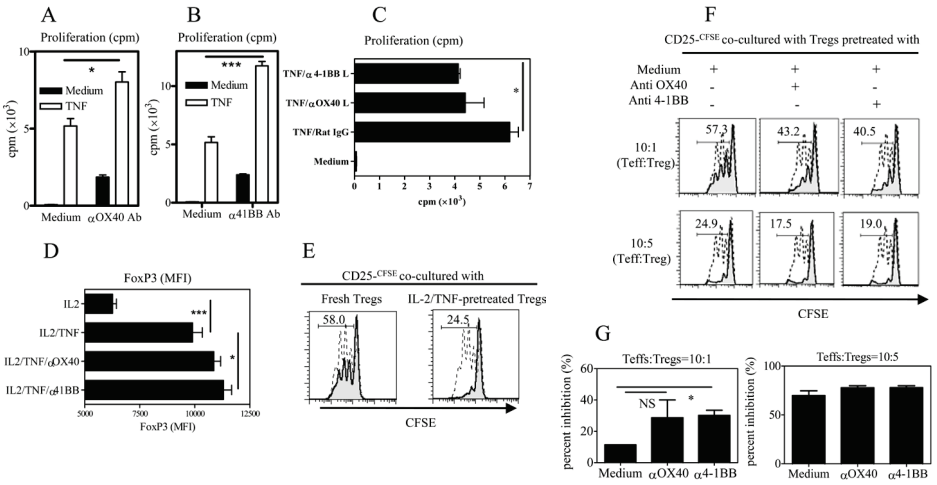

Figure 4. Activation of 4-1BB and OX40 expands potent suppressive Tregs. (A-B) $CD4^+FoxP3/gfp^+$ Tregs were stimulated with APCs and anti-CD3, with or without TNF, or with agonistic anti-OX40 Ab, A) or with agonistic anti-4-1BB Ab (B). After 72-hr incubation, proliferation was determined by [3]H thymidine incorporation assay. (C) $CD4^+FoxP3/gfp^+$ Tregs were stimulated with APCs and anti-CD3, with medium, or with TNF, or TNF plus blocking anti-OX40 L Ab or anti-4-1BB L Ab. After 72-hr incubation, proliferation was determined by [3]H thymidine incorporation assay. (C) $CD4^+FoxP3/gfp^+$ Tregs were cultured with IL-2 with or without TNF, or TNF plus agonistic anti-OX40 Ab, or anti-4-1BB Ab. After 3 days, MFI of FoxP3 expression was analyzed with FACS. Data (means ± SEM, n=3) shown in (A~D) are representative of three experiments with similar results. The comparison of two indicated groups, * $p<0.05$; *** $p<0.001$. (E) $CD4^+CD25^+$ Tregs were treated with medium which contained TNF/IL-2 for 3 days. After washing, pre-treated Tregs or freshly FACS-purified $CD4^+CD25^+$ Tregs were added into the freshly flow-sorted, CFSE-labeled $CD4^+CD25^-$ T cells at 10:5 ration (Teff:Treg). (F) $CD4^+CD25^+$ Tregs were treated with medium which contained TNF/IL-2, with or without agonistic anti-4-1BB Ab or anti-OX40 Ab for 3 days. After washing, pre-treated Tregs were added into the freshly flow-sorted, CFSE-labeled $CD4^+CD25^-$ Teffs at 10:1 and 10:5 ration (Teff:Treg). The cells were stimulated with APCs and anti-CD3 Ab. After 48-hr incubation, the proliferation was measured by CFSE dilution on Teffs with FACS. Dashed histogram: CFSE expression profile of Teffs alone; grey histogram: CFSE expression profile of Teffs co-cultured with Tregs. The number in the histogram stands for the proportion of CFSE-diluted cells in the presence of Tregs (%). Data shown are representatives of three separate experiments with similar results. (G) The summary of percent inhibition exerted by Tregs pretreated with medium alone (with TNF/IL-2) or with medium plus agonistic anti-OX40 Ab or anti-4-1BB Ab. Data shown (means ± SEM, n=6) were summarized from two separate experiments with similar results. Comparison with indicated two groups: *$p<0.05$. N.S., no statistical significance.

with anti-4-1BB Ab or anti-OX40 Ab retained and in the case of anti-4-1BB Ab could enhance their potent suppressive potential, as compared with Tregs pre-treated with TNF/IL-2 (Medium) alone ($p<0.05$, Fig 4F-G). Our data therefore indicate that up-regulation of 4-1BB and OX40 by TNF/IL-2 on Tregs could further promote their proliferation, while preserving or even enhancing their potent suppressive activity.

Neutralization of TNF blocks in vivo expansion of splenic Tregs after LPS challenge

It has been reported that LPS was able to activate and expand Tregs by interacting with TLR4 expressed on their surface (23). Since LPS is a potent inducer of TNF(24), we hypothesized that TNF produced in response to LPS challenge may also contribute to the LPS-induced expansion of Tregs. The results showed that, in vivo injection of LPS resulted in ~2-fold and >3-fold increase in the proportion of $FoxP3^+$ cells in the splenic $CD4^+$ subsets by 24 and 72 hrs after injection, respectively (Fig 5A). Similarly, the proportion of $FoxP3^+$ cells present in the draining mesenteric LN CD4 subset following intraperitoneal LPS injection was also increased from 8.54% in control mice to 14.24% (Fig 5B). The expansion of Tregs in CD4+ subset persisted until day 5 (data not shown). Moreover, the surface expression levels of TNFR2, 4-1BB and OX40 was markedly preferentially increased by 6 hrs on Tregs (Fig 5C). The up-regulation of these TNFRSF members on Tregs was transient, with a peak expression at 24 hrs for both TNFR2 and OX40, and 6 hrs for 4-1BB, respectively (Fig 5C). Thus, our data shows that in vivo administration of LPS also results in the activation and proliferation of Tregs. In order to confirm the role of TNF in the expansion of splenic Tregs, a neutralizing Ab against mouse TNF was injected 24 hrs and 1 hr before LPS challenge. The results showed that the neutralization of TNF markedly blocked the expansion of splenic Tregs ($p<0.001$, Fig 5D, E). Furthermore, expression of TNFR2, OX40 and 4-1BB on the splenic Tregs was also down-regulated by anti-TNF treatment (Fig 5F). Thus, TNF and TNFRSF contribute to the in vivo expansion of Tregs after LPS challenge.

Discussion

In this study, we for the first time report that TNF, in the presence of common gamma chain interleukins, had the capacity to up-regulate the expression of a number of co-stimulatory TNFRSF members, including its own receptor, TNFR2, as well as 4-1BB and OX40, preferentially on Tregs. This provides a means of amplifying Treg numbers to optimally attenuate the harmful excessive inflammatory responses.

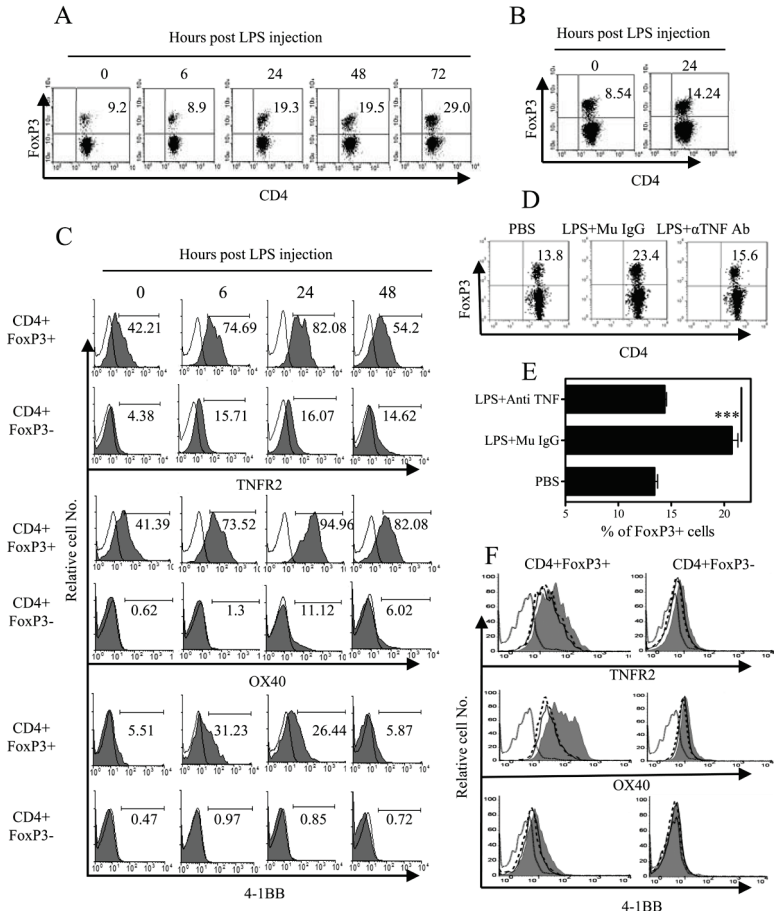

Figure 5. Neutralizing anti-TNF Ab blocked LPS-induced the expansion of Tregs and up-regulation of TNFRSFs on Tregs. Normal B6 mice were injected with 200 μg of LPS (i.p.) or PBS. Mouse spleens and mesenteric LNs were harvested at indicated time after injection. The proportion of $FoxP3^+$ cells present in the spleen (A) or mesenteric LNs (B) was analyzed with FACS, by gating on $CD4^+$ T cells. (C) Expression of TNFR2, OX40 and 4-1BB on Tregs or Teffs present in the spleen was analyzed with FACS, by gating on $CD4^+FoxP3^+$ cells or $CD4^+FoxP3^-$ cells. (D~F) Mice were i.p. injected with 200 μg of neutralizing anti-mouse TNF Ab (5E5) or control mouse IgG1 24 hrs and 1 hr before challenge of 200 μg of LPS or PBS (i.p.). On day 5 after LPS injection, the proportion of $FoxP3^+$ cells was analyzed with FACS, by gating on $CD4^+$ T cells. (D) Typical dot plot of FACS analysis. (E) The summary of percentage of $FoxP3^+$ cells in splenic CD4 subset (means ± SEM, n=5). Comparison of indicated two groups, *** $p<0.001$. (F) Expression of TNFR2, OX40 (24 hrs after LPS injection) and 4-1BB (6 hrs after LPS injection) was analyzed with FACS, by gating on $FoxP3^+$ CD4 cells. Grey: PBS treatment control; dashed line: isotype control; solid line: LPS and Mu IgG (left panel) or anti-TNF Ab (right panel) treatment. The number in the dot plot indicates the percentage of cells in the respective quadrants (%). The number in the histogram indicates the percentage of positive cells (%). Data shown are representative of three separate experiments with similar results.

TNF is not sufficient to support the in vitro survival of Tregs and thus either IL-2 or IL-7 was used. TNF and IL-2 up-regulate both TNFR2 and CD25 on Tregs, resulting in a reciprocal-amplification loop in the activation of Tregs. Although Tregs express low levels of IL-7 receptor α chain (CD127) which could not be up-regulated by TNF (data not shown), IL-7 and TNF nevertheless synergistically promoted the proliferative response of Tregs to TCR stimulation. In addition, TNF, in combination with IL-15, also activated Tregs (data not shown). The relative potency in support of Treg-activating effect of TNF were IL-2>IL-7>IL-15. Further, the effect of TNF/IL-7 or TNF alone on Tregs was not blocked by neutralizing anti-IL-2 Abs. Thus, the activating effects of both TNF and TNF/IL-7 on Tregs were not mediated by IL-2. The synergistic effects of TNF with other Cγ chain cytokines and TCR stimulation also likely contribute to the expansion and activation of Tregs at inflammatory site. We favor the idea that TNF-TNFR2 signaling pathway plays an important role in the activation of Tregs. A greater understanding of these fundamental mechanisms is needed for the discovery of novel approach to up- or down-regulate Treg activity at signal transduction and molecular levels.

4-1BB and OX40 are members of TNFRSF whose genes are clustered on mouse chromosome 4 together with TNFR2 (25). These molecules have some activities in common, such as regulating the expression of anti-apoptotic members of Bcl-2 family, promoting proliferation and survival of CD4 cells (21). The effects of these two molecules, especially of OX40, on the function of Tregs remain controversial. It has been reported that the anti-tumor effect of OX86, an agonistic antibody for OX40, was associated with attenuation of the suppressive function of Tregs (26). However, when used together with cyclophosphamide, OX86 actually induced the overactivation of tumor infiltrating Tregs, leading to selective apoptosis and eventual depletion of Tregs (27). It has been proposed that, if the “cytokine milieu is right”, OX40 agonist could promote Treg activity (20). Our data presented in this study indicate that TNF appear to be the “right cytokine” to provide activating effect of OX40 signaling on Tregs. Actually, OX40 signaling contributes to TNF-induced proliferative response of Tregs to APCs, since Treg proliferation was promoted by agonistic anti-OX40 Ab and partially abrogated by antagonistic anti-OX40 Ab (Fig 4A, C). This confirms a recent report of the contribution of OX40-OX40 ligand interaction to APC (DC)-mediated proliferation of Tregs (28). The physiological relevance of our findings is supported by the emerging evidence showing the crucial role of OX40 in the expansion, accumulation and function of Tregs in control of TNF-enriched inflammation, such as EAE (20) and colitis (29, 30). In fact, the stimulatory effects of OX40 and 4-1BB on Tregs have been harnessed in protocols aimed at expanding Tregs for

therapeutic purposes (19, 31) Thus, in addition to their known co-stimulatory effects on Teffs (21), OX40 and 4-1BB are also potent activators of Tregs.

Nagar and colleagues recently reported that stimulation with TNF up-regulated the transcription and surface expression of OX40 and 4-1BB in human Treg cells (15). However, they concluded that TNF decreased the suppressive activity of Tregs, based on their evidence that TNF stimulated the proliferation and cytokine production in co-cultures of Tregs and Teffs (15). Rather than decreasing Treg activity, their results can be attributed to the capacity of TNF to enhance the response of Teffs to TCR stimulation. Indeed, we have reported that TNF stimulated the activation of Teffs, which acquire the capacity to proliferate in spite of presence of Tregs in the early stage of co-culturing (3). Furthermore, TCR-activated mouse Teffs up-regulated their TNFR2 expression and become relatively resistant to suppression by Tregs (16). However, rather than impairing function of Tregs, TNF actually preferentially activated and expanded Tregs and eventually restored the suppression of co-cultures of mouse Tregs and Teffs (3). This viewpoint is favored by their data showing that the levels of TNF-induced INFγ in their Treg-Teff co-cultures paralleled the levels in unstimulated co-cultures (15), indicating that the degree of suppression by Tregs was not diminished by TNF. Nevertheless, we do not exclude the possibility that differences in species, experimental methods and time frame of observation may also contribute to the discrepancy between our data ((3) and this study) and Nagar et al's data (15) regarding the impact of TNF on the inhibition of proliferation in co-cultures.

The evidence that inflammatory responses can actually drive the proliferative expansion as well as enhancing the suppressive activity of Tregs is compelling, and is compatible with our conclusion that the interaction of TNF and TNFR2 promote both proliferation and suppressive activities of Tregs (32). Although counterintuitive and contradictory to most previous reports, our finding that TNF has the capacity to activate and expand Tregs has been supported by more recent studies. For example, TNF-TNFR2 interaction has been shown to be crucial for the generation and function of human $CD4^+$ as well as $CD8^+$ Tregs (8, 9), as well as to promote survival of human Tregs in an inflammatory environment by inducing anti-oxidative thioredoxin-1 (11). Interestingly, pathogenic Teff cell-derived TNF had the capacity to boost Treg activity in vivo and consequently suppressed autoimmunity in a mouse model (12).

Overall, our data indicate that, in concert with a common gamma chain cytokine (IL-2, IL-7 or IL-15), TNF preferentially up-regulates the expression of co-stimulatory members of TNFRSF such as TNFR2, 4-1BB and OX40 on Tregs, resulting in a positive feedback

amplification of the stimulatory effect of TNF on Tregs. Thus, TNF enhances multiple TNFRSF pathways by up-regulating a number of receptors that can cooperate to curtail excessive inflammation and prevent self destructive tissue damage.

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Chapter 6

TNFR2 is critical for the stabilization of the $CD4^+Foxp3^+$ regulatory T cell phenotype in the inflammatory environment

Xin Chen, Xueqiang Wu, Qiong Zhou, O.M. Zack Howard, Mihai G. Netea and Joost J. Oppenheim

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Abstract

Several lines of evidence indicate the instability of $CD4^+Foxp3^+$ regulatory T cells (Tregs). We have therefore investigated means of promoting the stability of Tregs. In this study, we found that the proportion of Tregs in mouse strains deficient in TNFR2 or its ligands was reduced in the thymus and peripheral lymphoid tissues, suggesting a potential role of TNFR2 in promoting the sustained expression of Foxp3. We observed that upon in vitro activation with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab, Foxp3 expression by highly purified mouse Tregs was markedly down-regulated. Importantly, TNF partially abrogated this effect of TCR stimulation and stabilized Foxp3 expression. This effect of TNF was blocked by anti-TNFR2 Ab, but not by anti-TNFR1 Ab. Furthermore, TNF was not able to maintain Foxp3 expression by TNFR2-deficient Tregs. In mouse colitis model induced by transfer of naïve CD4 cells into $Rag1^{-/-}$ mice, the disease could be inhibited by co-transfer of WT Tregs, but not by co-transfer of TNFR2-deficient Tregs. Furthermore, in the lamina propria of the colitis model, the majority of WT Tregs maintained Foxp3 expression. In contrast, increased number of TNFR2-deficient Tregs lost Foxp3 expression. Thus, our data clearly show that TNFR2 is critical for the phenotypic and functional stability of Treg in the inflammatory environment. This effect of TNF should be taken into account when designing future therapy of autoimmunity and GVHD by using TNF inhibitors.

Introduction

$CD4^+Foxp3^+$ regulatory T cells (Tregs) play a nonredundant role in the maintenance of immunological homeostasis and in the prevention of autoimmune disorders, and they also represent a major cellular mechanism in immune evasion by tumors (1, 2). It is now well established that the transcription factor Foxp3 (forkhead box P3) is a unique marker specific for the Treg lineage, which determines their phenotype and immunosuppressive function (3). Abrogation of Foxp3 gene function leads to the development of lethal multi-organ autoimmune disorders in mice and humans (4, 5). In contrast, forced expression of Foxp3 in $CD4^+CD25^-$ effector T cells (Teffs) is sufficient to convert them into functional Tregs (6, 7).

Contrary to the previous notion that Tregs were stable and terminally differentiated cells, recent evidence revealed the phenotypic and functional plasticity of Tregs in response to inflammatory stimulation. For example, IL-1β and IL-6 each re-programmed Treg cells and induced them to express IL-17 (8-10). However, there is compelling contrasting evidence that the number of highly suppressive Foxp3-expressing cells actually increased in various inflammatory sites (11). Therefore, the inflammatory environment seems to have the capacity to restrain the plasticity and promote the phenotypic and functional stability of Tregs. Clarification of the molecular basis of such an effect may be therapeutically beneficial and can further improve our understanding of Treg biology.

TNF is a pleiotropic cytokine and up-regulation of TNF expression is a benchmark of inflammatory responses. The biological functions of TNF are mediated by two structurally related, but functionally distinct receptors, TNFR1 (tumor necrosis factor receptor type I or p55) and TNFR2 (tumor necrosis factor receptor type II or p75) (12). With a death domain (DD) in its cytoplasmic tail, TNFR1 is the primary signaling receptor on most cell types and accounts for the majority of the proinflammatory, cytotoxic and apoptotic effects classically attributed to TNF (13, 14). In contrast, TNFR2 lacks an intracellular death domain and predominantly mediates signals promoting lymphocyte activation and proliferation (15, 16). Although counterintuitive, the accumulated evidence indicates that TNF by signaling through TNFR2 promotes Treg activity. For example, we reported that TNF by activating TNFR2 is able to activate and expand Tregs (17), and TNFR2 expression identifies the maximal suppressive (18) and replicating Tregs (19) in mice and helps identify functional Tregs in human PBMCs (20). Furthermore, TNF preferentially up-regulates TNFR2 expression on Tregs (21). In confirmation of our observation, Housley and Clark et al have shown that TNFR2 is critical for the in vivo immunosuppressive

function of naturally occurring Tregs (22). Grinber-Bleyer and Salomon et al showed that pathogenic Teffs stimulated the activation of Tregs in vivo, at least partially through TNF-TNFR2 interaction (23). Mougiakakos and Kiessling et al found that TNF-TNFR2 interaction enhanced thioredoxin-1 expression on human Tregs, but not on Teffs, and therefore preferentially promoted Treg survival and activity within inflammatory milieu (24). However, it remains elusive whether the replicating Tregs driven by TNF-TNFR2 interaction retain phenotypic and functional attributes of naturally occurring Tregs, especially in the chronic inflammatory condition. This question is clinically relevant now that TNF blockage therapy has become an important tool in the treatment of autoimmune disorders.

In this study, we observed that the frequency of Tregs in the thymus of mouse strains genetically deficient in TNFR2 or its ligands is reduced. Expression of Foxp3 is induced in developing thymocytes upon self-reactive TCR engagement (25). Thus, stimulation of TNFR2 may stabilize Foxp3 expression in response to activation of TCR of Treg cells in immune or inflammatory responses, as shown by our in vitro and in vivo studies.

Materials and Methods

Mice and reagents. Wild-type C57BL/6 mice, congenic Ly5.2 C57BL/6 mice, $Rag1^{-/-}$ mice, $TNFR2^{-/-}$ mice, $TNF^{-/-}$ mice, $TNN/LT\alpha/LT\beta^{-/-}$ mice were provided by the Animal Production Area of the NCI (Frederick, MD). Foxp3/gfp KI (knock in) mice were kindly provided by Dr. Yasmine Belkaid at Laboratory of Parasitic Diseases, NIAID, NIH, and maintained in the Animal Production Area of the NCI (Frederick, MD). Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.). Anti-mouse antibodies (Abs) were purchased from BD Biosciences (San Diego, CA) consisted of anti-mouse CD3 (145-2C11), CD4 (GK1.5), CD25 (PC61), CD45, TNFR2 (TR75-89), Ki-67 (B56), INFγ (XMG1.2) and IL-17A (TC11-18H10). Leukocyte Activation Cocktail was also purchased from BD Biosciences. Functional grade purified anti-mouse CD3e (eBio500A2), CD28 (37.51) and IL-4 (11B11) Abs, Foxp3 Staining Set (FJK-16s) and anti-mouse TCRβ Ab (H57-597) were purchased from eBioscience (San Diego, CA). Functional grade anti-mouse TNFR1 (55R-170), TNFR2 (TR75-32.4) Ab and Ham IgG were purchased from Biolegend (San Diego, CA). Murine

IL-6, IL-12 and TNF were purchased from PeproTech (Rocky Hill, NJ). Human rTGFβ1 was from R&D Systems (Minneapolis, MN).

T cell transfer model of colitis. Naive $CD4^+CD25^-CD45RB^{hi}$ T cells were isolated from WT congenic B6 ($CD45.1^+$) mice and injected i.p. into $Rag1^{-/-}$ immunodeficient recipients (4×10^5 cells/mouse) alone, or co-transferred with WT or $TNFR2^{-/-}$ $CD4^+CD25^+CD45RB^{lo}$ Treg cells (1.6×10^5/mouse, $CD45.2^+$). In Tregs transfer experiments, $CD4^+CD25^+CD45RB^{lo}$ Treg cells flow-sorted from WT congenic B6 mice (CD45.1+) and $TNFR2^{-/-}$ mice ($CD45.2^+$) were mixed at 1:1 ratio and i.p. injected into $Rag1^{-/-}$ mice (1.2×10^5/mouse, each). Mice were monitored weekly for the clinical symptoms of colitis such as rectal bleeding, loose feces/diarrhea, rough/hunched posture and body weight by animal facility staff. Any mice losing >20% of its starting body weight or showing severe signs of disease were euthanized.

*Cell isolation***.** Single cell suspensions from spleen and mLNs (mesenteric LNs) were prepared by filtration through a 70 μm cell strainer (BD Labware, San Jose, CA). Preparation of colon lamina propria (cLP) cells was as previously described (26). Briefly, colons were rinsed in PBS and cut into ~0.3 cm pieces. Intestinal epithelial cells were removed by incubation with Ca- and Mg-free PBS containing 10% FCS and 5 mM EDTA. Colon tissues then were incubated with RPMI 1640 containing 10% FCS and 1 mg/ml collagenase type 4 (Worthington Biochemical Corporation, Lakewood, NJ) for 30 min at 37°C.

In vitro T cell activation and differentiation. Flow-sorted $CD4^+Foxp3/gfp^+$ cells or $CD4^+Foxp3/gfp^-$ cells from Foxp3/gfp KI mice, or $CD4^+CD25^+$ cells from WT C57BL/6 mice or $TNFR2^{-/-}$ mice were seeded at 5×10^4 cells/well in 96-well plate. The cells were stimulated with plate-bound anti-CD3e Ab (10 μg/ml) and soluble anti-CD28 Ab (2 μg/ml) for 5 days. In some experiments, the cells were activated in Th0, Th1 and Th17 polarizing conditions by adding medium alone, or IL-12 (10 ng/ml) plus anti-IL-4 Ab (10 μg/ml), or IL-6 (10 ng/ml) or IL-6 plus TGFβ (1 ng/ml), respectively. In some experiment, the cells were activated in the presence of TNF (10 ng/ml) and/or IL-6 (10 ng/ml), without or with 10 μg/ml of Ham IgG, or anti-TNFR1 Ab or anti-TNFR2 Ab. Iscove's modified Dulbecco's medium (IMDM, Sigma-Aldrich) was used in Th17 polarizing culture, and RPMI-1640 (Lonza BioWhittaker, Walkersville, MD) was used in all other cultures. The medium was supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 50 μM 2-ME.

Flow cytometry. After blocking FcR, cells were incubated with appropriately diluted antibodies. Acquisition was performed using a SLRII (BD Biosciences, Mountain View, CA) and data analysis was conducted using FlowJo software (Tree Star Inc., Ashland, OR). For intracellular cytokines staining, cells were re-stimulated with BD Leukocyte Activation Cocktail for 4 h. FACS analysis was gated on the live cells only by using LIVE/DEAD® Fixable Dead Cell Stain Kit.

Statistical analysis. Cumulative incidence of colitis was graphed as survival plot and analyzed with Logrank test, and comparison of other data was analyzed by two-tailed Student's *t* test using Graphpad Prism 4.0.

RESULTS

Reduction of thymic and peripheral Tregs in mice deficient in TNFR2 or its ligands

In normal mice, most thymic Tregs express TNFR2 (18). All human thymic $CD4^+CD25^+$ Tregs constitutively express TNFR2, while thymic $CD4^+CD25^-$ cells do not express this receptor (27). TNF is expressed in the thymus of mice and humans, and participates in the development of thymocytes (28). Thus, we investigated the possibility that TNF or LTα (lymphotoxin alpha), the ligands for TNFR2, contribute to the thymic differentiation and generation of Tregs.

We first compared the Foxp3-expressing Tregs in $TNFR2^{-/-}$ and normal WT mice. In adult $TNFR2^{-/-}$ mouse thymus, the proportion of $CD4^+Foxp3^+$ Tregs in total thymocytes was reduced by 45%, as compared with WT control B6 mice (Fig 1A, $^*P<0.05$). The proportion of $Foxp3^+$ cells in CD4 SP (single positive) thymocytes was also reduced by ~30% (Fig 1B, $p<0.05$). It was reported that the cellularity of the thymus of $TNFR2^{-/-}$ mice was greater than that of WT mice, however, the most affected subset of thymocytes were naïve triple negative cells ($CD3^-CD4^-CD8^-$), while both CD4 and CD8 subsets were not altered (29). Since thymic Tregs were almost exclusively contained in the CD4 SP population, the absolute number of Tregs in the thymus of $TNFR2^{-/-}$ mice was reduced proportionally.

In the periphery, the percent of $CD4^+Foxp3^+$ cells in total splenic cells and LN cells was decreased by 42% and 21%, respectively ($P<0.01$~0.05, Fig 1C~D). The proportion of $Foxp3^+$ cells in the $CD4^+$ splenic cells and $CD4^+$ LN cells was decreased by 36% and 22%, respectively ($p<0.05$, Fig 1E~F). The absolute number of splenic Tregs in $TNFR2^{-/-}$ mouse was reduced by ~50% ($p<0.05$, Fig 1G). These data suggest that TNFR2 may participate in the development of Tregs in the thymus. Although $TNFR2^{-/-}$ mouse does not spontaneously develop apparent autoimmune disorders, this strain of mouse nevertheless shows more

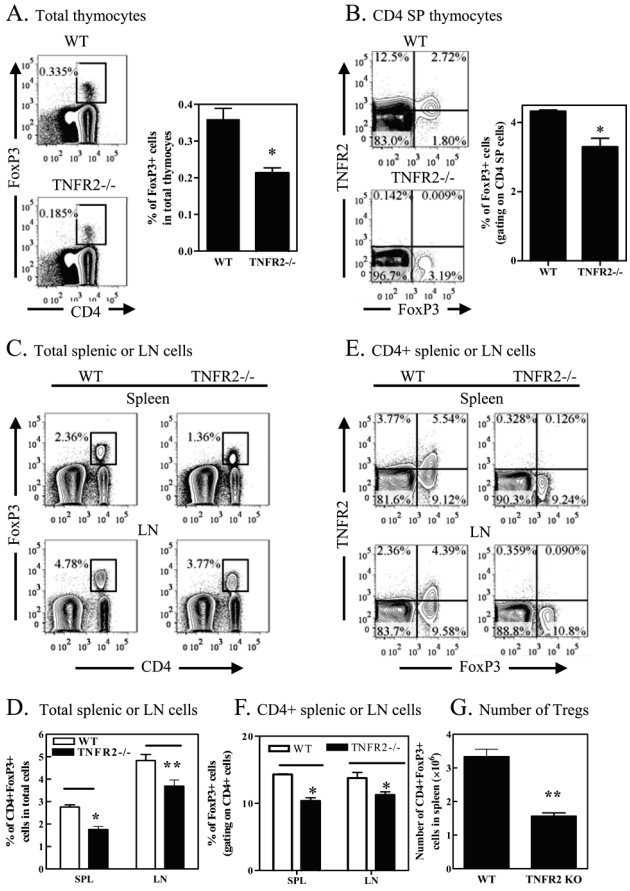

Figure 1. **Reduced number of Tregs in TNFR2 deficient mice.** Cells from thymus, spleen and LNs in WT mice (C57BL/6) and TNFR2$^{-/-}$ mice were stained with CD3, CD4, CD8, TNFR2, and Foxp3. The expression of Foxp3 was analyzed by FACS, gating on CD3^{+}CD4^{+} cells or CD3^{+}CD4^{+}CD8^{-} cells (CD4 single positive cells, e.g., CD4 SP cells). (A) Proportion of CD4^{+}Foxp3^{+} cells in the total thymocytes derived from WT or TNFR2$^{-/-}$ mice. (B) Expression of Foxp3 and TNFR2 on CD4 SP thymocytes from WT or TNFR2$^{-/-}$ mice. (C~D) Proportion of CD4^{+}Foxp3^{+} cells in the total splenic and LN cells from WT or TNFR2$^{-/-}$ mice. In (A and B), left shows the typical FACS plots, and right shows summary (N=3). (C) shows the typical FACS plots and (D) shows the summary (N=3). (E-F) Expression of Foxp3 and TNFR2 on CD4^{+} T cells in the spleen and LNs from WT or TNFR2$^{-/-}$ mice. (E) Shows the typical FACS plots and (F) shows the summary (N=3). (G) Number of CD4^{+}Foxp3^{+} Tregs in the spleen from WT or TNFR2$^{-/-}$ mouse. Number in the FACS plot shows the proportion of positive cells in the indicated gating or respective quadrants. Comparison between two indicated groups: * $p<0.05$; ** $p<0.01$. Data shown are representatives of at least 3 separate experiments with same results.

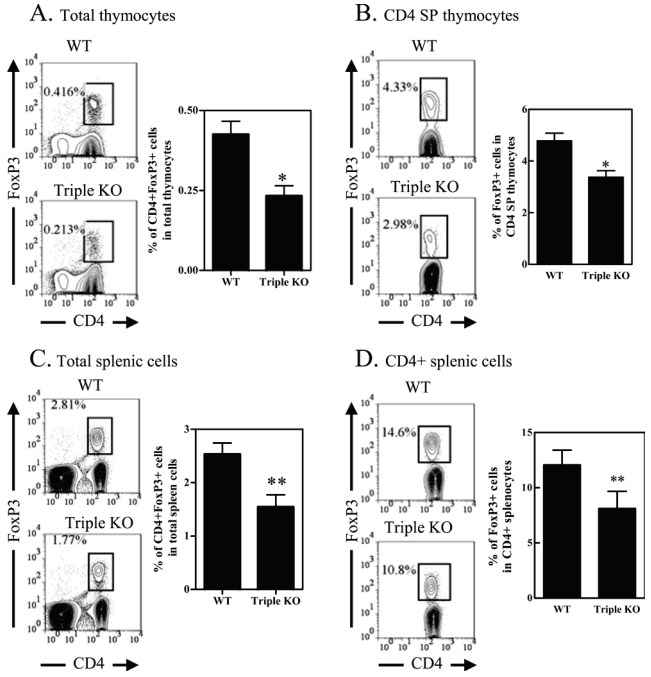

Figure 2. Reduced number of Tregs in TNFR2 ligands deficient mice. Cells from thymus, spleen and LNs in WT mice (C57BL/6) or TNF/LTα/LTβ triple KO mice were stained with CD3, CD4, CD8, TNFR2, and Foxp3. The expression of Foxp3 was analyzed by FACS, gating on $CD3^+CD4^+$ cells or $CD3^+CD4^+CD8^-$ cells. (A) Proportion of $CD4^+Foxp3^+$ cells in total thymocytes from WT or Triple KO mice. (B) Proportion of $Foxp3^+$ cells in the $CD4^+CD8^-$ thymocytes from WT or Triple KO mouse. In (A and B), left shows the typical FACS plots, and right shows summary (N=3~6). (C) Proportion of $CD4^+Foxp3^+$ cells in the spleen from WT or Triple KO mice. (D) Proportion of $Foxp3^+$ cells in $CD4^+$ splenic cells from WT or Triple KO mice. Number in the FACS data shows the proportion of cells in the indicated gating. Comparison between two indicated groups: * $p<0.05$; ** $p<0.01$. Data shown are representative of at least 3 separate experiments with same results.

severe inflammation upon induction of autoimmune disease (30), presumably attributed by the reduced number of Tregs.

The development of $Foxp3^+$ Tregs in mice with depletion of TNFR2 ligands was also investigated. Both $TNF^{-/-}$ mice or $LT\alpha/\beta^{-/-}$ mice did not exhibit any deficiency in Tregs in the spleen (data not shown). However, the proportion of Tregs in the periphery and thymus of $TNF/LT\alpha/LT\beta^{-/-}$ (triple KO) mice was decreased. Despite a profound defect of peripheral lymphoid organs of triple KO mice, this strain of mouse had no change in the major thymocyte populations and T/B ratio in the spleen (31). In the thymus, the proportion of

$CD4^+Foxp3^+$ Tregs in total thymocytes was reduced by ~50% ($p<0.05$, Fig 2A). The proportion of $Foxp3^+$ Tregs in CD4 SP thymocytes was reduced by 31% ($p<0.05$, Fig 2B). In the spleen, the proportion of $CD4^+Foxp3^+$ Tregs in total splenic cells was reduced by 37% ($p<0.01$, Fig 2C). The proportion of $Foxp3^+$ Tregs in $CD4^+$ splenic cells was reduced by 26% ($p<0.01$, Fig 2D). TNF treatment in vitro largely restored the proportion of $Foxp3^+$ Tregs in CD4 cells derived from triple KO mice (data not shown). Therefore, genetic ablation of TNFR2 or its ligands resulted in a reduction of Tregs in both thymus and peripheral lymphoid tissue.

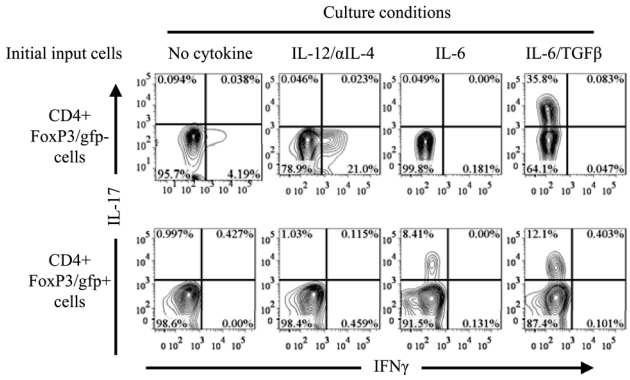

Figure 3. Plasticity of Tregs under proinflammatory stimulation in vitro. Splenic and LN cells from Foxp3/gfp KI mice were stained with CD4. $Foxp3/gfp^+$ or $Foxp3/gfp^-$ CD4 cells were flow-sorted. The cells were activated in vitro with plate-bound anti-CD3 and soluble anti-CD28 Abs, in Th0 (no cytokine added) or in Th1 (IL-12 plus anti-IL-4 Ab) or Th17 (IL-6, or IL-6 plus TGFβ) polarizing culture condition. After 5 days, the cells were re-stimulated with BD Leukocyte Activation Cocktail and intracellular IFNγ and IL-17A were analyzed by FACS. Numbers in FACS plots indicate the percentage of cells in the respective quadrant. The data shown are representative of at least three separate experiments with the same results.

TNF stabilizes Foxp3 expression by TCR-stimulated Tregs in vitro

Self-reactive TCR signaling plays a central role in the thymic generation of Tregs (25). Reduced thymic Tregs in mice deficient in TNFR2 and its ligands suggest that TNF-TNFR2 signaling may play a role in stabilizing Foxp3 expression by Tregs in response to TCR stimulation. We therefore examined the effect of exogenous TNF on Foxp3 expression by highly purified Tregs, which were stimulated in vitro with plate bound anti-CD3 Ab and soluble anti-CD28 Ab. It was reported that, in the presence of proinflammatory cytokine, in vitro TCR stimulation could change the phenotype of Tregs (30). We confirmed this result and found that IL-6+TGFβ induced IL-17 expression by both Tregs and Teffs (effector T cells), IL-6 alone only induced IL-17 expression by TCR-stimulated Tregs, but not by Teffs. In contrast, Th1 polarizing culture condition only induced IFNγ expression by TCR-stimulated Teffs, but not by Tregs (Fig 3). Unexpectedly, TCR-stimulation alone markedly down-regulated Foxp3 expression by Tregs (Fig 4). This was not caused by activation induced cell death (AICD), since FACS analysis only gated on

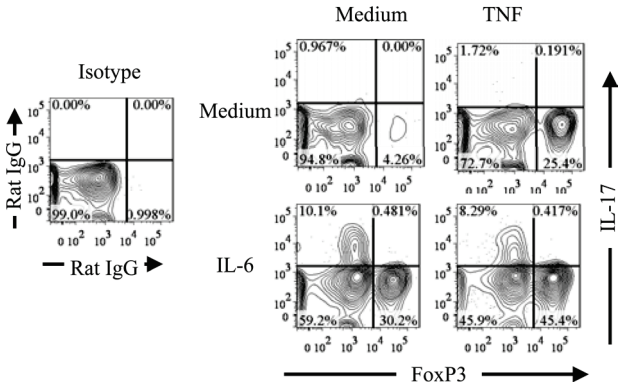

Figure 4. TNF stabilizes Foxp3 expression on Tregs in vitro. Flow-sorted CD4$^+$Foxp3/gfp$^+$ cells were stimulated as described in Fig 3, with medium alone or with IL-6, in the presence of TNF or not. After re-activation, intracellular expression of Foxp3 and IL-17A was analyzed with FACS. Numbers indicate the percentage of cells in the respective quadrant. The data shown are representative of at least three separate experiments with the same results.

the live cells by using LIVE/DEAD® Fixable Dead Cell Stain Kit described in Methods and Materials. Although IL-6 induced IL-17 expression by a substantial proportion of initial Tregs, which all down-regulated their Foxp3 expression, this proinflammatory cytokine paradoxically supported the persistent expression of Foxp3 by 30% of input cells (Fig 4). In contrast, TNF by itself consistently maintained high levels of Foxp3 expression by 20~30% of initial input Tregs, and can cooperate with IL-6 to further enhanced the proportion of Foxp3-expressing cells without further increasing IL-17-producing cells (Fig 4). Therefore, in addition to activate and expand Tregs (17), TNF can also stabilize Foxp3 expression on Tregs.

TNFR2 mediates the effect of TNF in maintenance of Foxp3 expression in vitro

To further determine which TNF receptor was responsible for stabilizing Foxp3 expression, anti-TNFR1 or anti-TNFR2 neutralizing antibodies were added to the TNF-treated Tregs. The anti-TNFR2 Ab, but not anti-TNFR1 Ab, markedly reduced the proportion of Foxp3-expressing cells ($p<0.05$, Fig 5A). We further clarified this issue by comparing the response of Tregs from WT mice or TNFR2$^{-/-}$ mice. Although TNFR2$^{-/-}$ mice had reduced number of Tregs, the residual Tregs nevertheless expressed the same levels of Foxp3 as WT Tregs (Fig 5B). As shown in Fig 5C, TCR stimulation reduced Foxp3 expression by TNFR2$^{-/-}$ Tregs and this was not reversed by TNF. In contrast, Foxp3 expression by TNFR2$^{-/-}$ Tregs was partially sustained by IL-6 (data not shown). Thus, our data clearly show that TNF activation of TNFR2 is able to stabilize Foxp3 expression by Tregs upon potent TCR stimulation.

TNFR2 is required for the in vivo immunosuppressive function of Tregs

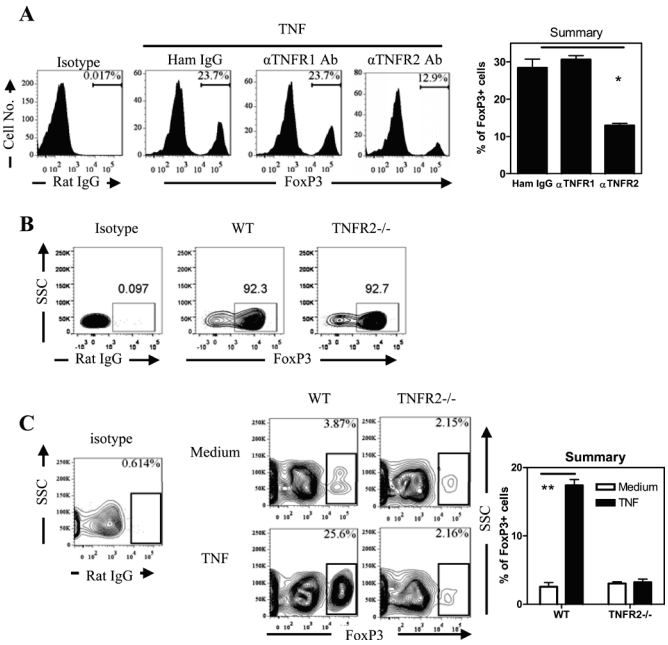

Figure 5. TNFR2 mediates the effect of TNF in maintaining Foxp3 expression in vitro. (A) Flow-sorted $CD4^+Foxp3/gfp^+$ cells were stimulated as in Fig 3, in the presence of TNF (10 ng/mL), and 10 μg/mL of isotype control Ham IgG, or anti-TNFR1 Ab or anti-TNFR2 Ab. Foxp3 expression was analyzed by FACS. Typical histograms and summary (N=3) are shown. (B) Tregs were flow-sorted from WT and $TNFR2^{-/-}$ mice, based on surface expression of $CD4^+CD25^+$. The expression of Foxp3 was analyzed by FACS. (C) $CD4^+$ $CD25^+$ cells from TNFR2 KO mice were activated as described in Fig 3, in the presence of TNF (10 ng/mL) or not. The expression of Foxp3 was analyzed by FACS. Typical FACS plot and summary (N=3) are shown. Numbers represent the percentage of cells within the indicated gate. The data shown are representative of at least three separate experiments with the same results. Compared with indicated group, * $p<0.05$; ** $p<0.01$.

The critical role of functional Tregs in immune homeostasis can be demonstrated in a mouse colitis model induced by transfer of naïve CD4 T cells into $Rag1^{-/-}$ mice (26). In a preliminary experiment, we found that eight weeks after transfer, TNF can be expressed by both host cells and adoptively transferred cells in the cLP (data not shown), while TNFR2 expression was up-regulated by both co-transferred initial naïve CD4 cells and Tregs (data not shown). Thus, this model is appropriate to determine the role of interaction of TNF-TNFR2 on the function of Tregs. We confirmed that TNFR2 expression on Tregs was required for the suppression of colitis induced by transfer of naïve CD4 cells (22) (Fig 6A, $p<0.05$). Furthermore, we compared the effect of WT or TNFR2-deficient Tregs on the development of Th1 or Th17 responses by co-transferred naïve CD4 cells. As shown in Fig 6B, naïve CD4 cells transferred alone (without Tregs) into $Rag1^{-/-}$ mice could develop into

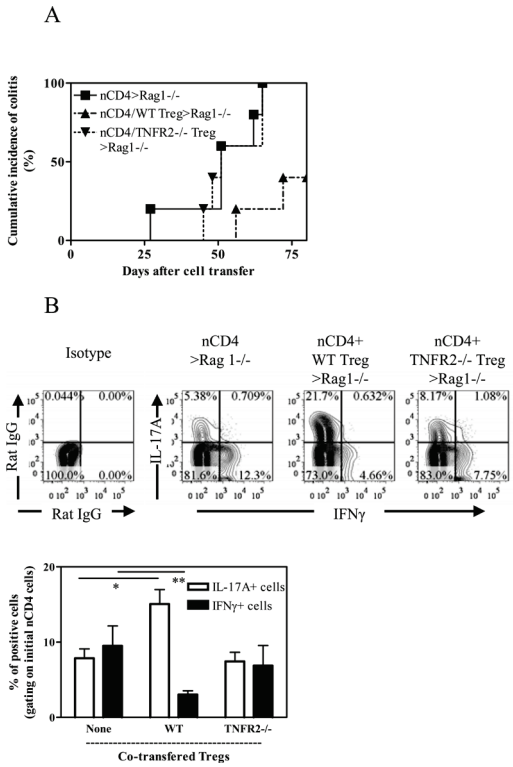

Figure 6. TNFR2 is required for the immunosuppressive function of Tregs in vivo. CD45.1$^+$ CD4$^+$CD25$^-$CD45RB^{hi} naïve T (nCD4) cells were transferred alone or co-transferred with CD45.2$^+$ WT or TNFR2$^{-/-}$ Treg cells into Rag1$^{-/-}$ mice. (A). Cumulative incidence of colitis. Incidence of colitis in mice co-transferred with naïve CD4 cells and WT Tregs was markedly decreased, by comparison with mice transferred with naïve CD4 cells alone or co-transferred with naïve CD4 cells and TNFR2$^{-/-}$ Tregs (p=0.0132 and 0.0163, respectively). (B) After eight weeks, cLP cells were isolated. The intracellular expression of IFNγ and IL-17A by initial transferred naïve WT CD4 cells was analyzed by FACS, gating on CD45.1$^+$ cells. Typical FACS plot and summary (N=3~5) are shown. Data shown are representative of three separate experiments with similar results.
* p<0.05; ** p<0.01.

both Th1 and Th17 cells in the colon, as indicated by their expression of IFNγ and IL-17A. Consistent with a previous report (22), WT Tregs markedly inhibited a proportion of IFNγ-producing cells (p<0.01), while TNFR2$^{-/-}$ Tregs failed to do so. It has been shown that, in mouse colitis model induced by transfer of naïve CD4 cells, Th17 cells had paradoxically tissue protective and immunosuppressive effect and consequently suppressed colon inflammation, by inhibiting pathogenic Th1 responses (31) or by enhancing barrier function of intestinal epithelial cells (32). In agreement with a number of recent studies (33, 34), we also found that transfer of WT Tregs resulted in greater than a 2-fold increase in the proportion of IL-17A-producing cells developed from the initial naïve CD4 cells, as compared with naïve CD4 cells transfer alone (p<0.01). In contrast, co-transfer of TNFR2-deficient Tregs failed to promote the generation of Th17 cells (p>0.05, Fig 6B). Therefore, TNFR2 is required for Tregs to suppress pathogenic Th1 response in this model.

TNFR2 is required for maintenance of Foxp3 expression on Tregs in vivo

The accumulation of Tregs and their Foxp3 expression were examined when full-fledged colitis was developed which occurred typically 6 to 8 weeks after cell transfer. As shown in Fig 7A, the proportion of WT Tregs present in the total numbers of transferred cells was

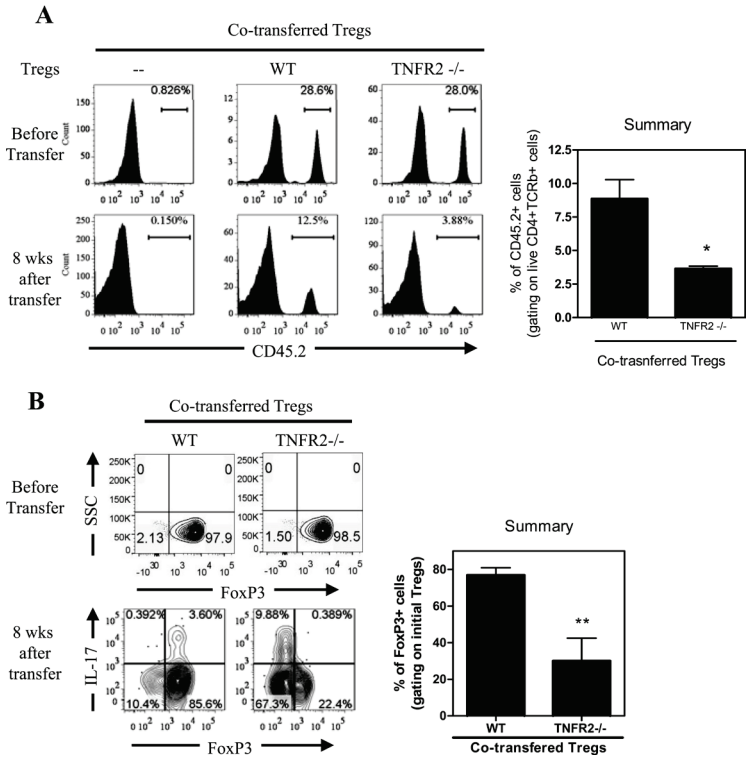

Figure 7. **Critical role of TNFR2 in stabilizing Foxp3 expression in vivo**. CD45.1^{+} CD4^{+}CD25^{-}CD45RB^{hi} naïve T cells were transferred alone or co-transferred with CD45.2^{+} WT or TNFR2$^{-/-}$ Treg cells into Rag1$^{-/-}$ mice. After eight weeks, cLP cells were analyzed by FACS. (A) Reduced relative number of co-transferred TNFR2$^{-/-}$ Tregs. Proportion of CD45.2^{+} initial Treg cells in total transferred CD4 cells was determined. For comparison, pre-transferred cells were shown in the upper panel. (B) Reduced Foxp3 expression by initial Tregs from TNFR2$^{-/-}$ mice in cLP. Expression of IL-17A and Foxp3 was analyzed by FACS, by gating on CD45.2^{+} initial Tregs. Numbers in the FACS plots represent the percentage of cells in the indicated gate or quadrant. Typical FACS plot and summary (N=3~5) are shown. Data shown are representative of three separate experiments with similar results. * $p<0.05$; ** $p<0.01$.

reduced from 28.6% to 12.5%, which may be based on the homeostatic restoration of Tregs to the normal 10~15% range in total CD4 pool. However, the proportion of Foxp3^{+}TNFR2$^{-/-}$ Tregs was reduced to 3.88%, which is markedly lower than 12.5% WT Tregs ($p<0.05$).

Flow-sorted Tregs from WT and TNFR2$^{-/-}$ mice expressed the same high levels of Foxp3 (Fig 7B). By 8 weeks after transfer, the majority of WT Tregs (>80%) maintained their Foxp3 expression in cLP. However, Foxp3 expression by TNFR2-deficient Tregs was markedly reduced (~40%), as compared with WT Tregs ($p<0.01$, Fig 7B). The reduction of

Foxp3 by TNFR2$^{-/-}$ Tregs was more profound in the cLP, while there was not a significant change in the spleen and mesenteric lymph nodes (data not shown), suggesting a role of proinflammatory response in the colon. Therefore, TNFR2 expression is critical for the phenotypic stability of Tregs in the inflammatory environment and for the capacity of Tregs to compete efficiently with colitogenic T cells. The latter effect may be attributable to the lack of suppressive function of TNFR2-deficient Tregs in vivo found in our study and others (22, 35).

TNFR2 expression is critical for the expansion of Tregs in competitive environment

The magnitude of inflammation was greater in Rag1$^{-/-}$ mice co-transferred with WT naïve CD4 cells containing TNFR2$^{-/-}$ Tregs, which might be attributable to the profound loss of Foxp3 expression in TNFR2$^{-/-}$ Tregs. To clarify this issue, we injected a mixture of WT and TNFR2$^{-/-}$ Tregs into Rag1$^{-/-}$ mice, in order to compare phenotypic stability of WT and TNFR2$^{-/-}$ Tregs in vivo in the identical environment. Tregs were flow-sorted from CD45.1^{+} WT and CD45.2^{+} TNFR2$^{-/-}$ mice and transferred at a 1:1 ratio into Rag1$^{-/-}$ mice (Fig 8A). Purified Tregs from these two strains of mice expressed the same high levels of Foxp3 (Fig 8B). Ten weeks after transfer, the proportion of WT Tregs to TNFR2$^{-/-}$ Tregs was changed from 1:1

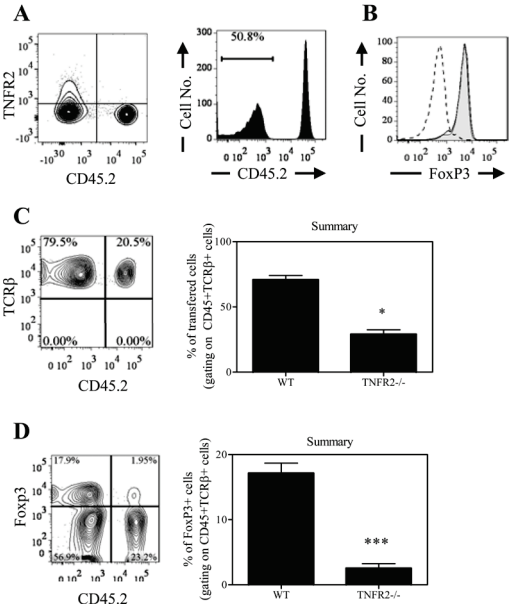

Figure 8. **TNFR2 expression is critical for the expansion of Tregs in competitive environment**. CD45.1^{+} Tregs and CD45.2^{+} Tregs were flow-sorted from WT mice and TNFR2$^{-/-}$ mice respectively and co-transferred into Rag1$^{-/-}$ mice at ratio of 1:1. (A) Profile of pre-transferred Tregs. Left shows expression of TNFR2 and CD45.2 and right shows CD45.2 expression alone. (B) Flow-sorted Tregs from WT mice (solid histogram) and TNFR2$^{-/-}$ mice (grey histogram) express comparable levels of Foxp3. Dashed line: isotype control. (C) Ten weeks after transfer, Tregs present in the cLP were analyzed by FACS, gating on CD45^{+}TCRβ^{+} cells. Left: expression of TCRβ and CD45.2 on cLP Tregs; right: summary of proportion of WT and TNFR2$^{-/-}$ Tregs in total transferred cells present in cLP (N=5). (D) Foxp3 expression on initial Tregs from WT or TNFR2$^{-/-}$ mice co-transferred into Rag1$^{-/-}$ mice (left) and summary of proportion of Foxp3-expressing cells in total transferred cells (right, N=5). The number shown in the FACS plots represent the proportion of cells in the respective quadrant or gate. Data shown are representatives of three separate experiments with similar results. * $p<0.05$; *** $p<0.001$.

to roughly 4:1 (Fig 8C, $p<0.05$), indicating a greater capacity of WT Tregs than TNFR2$^{-/-}$ Tregs to re-constitute the lymphopenic environment (Fig 8C). The majority of WT Tregs lost their Foxp3 expression and <40% expressed Foxp3 (Fig 8D), which is much lower than Tregs co-transferred with naïve CD4 cells (Fig 7B). These data are consistent with previous studies showing that a majority of transferred Foxp3/gfp^{+} Tregs in cLP of recipient lymphopenic mice lose their Foxp3 expression, while co-transfer of Teffs resulted in maintaining Foxp3 expression by Tregs (36, 37), presumably by producing cytokines such as IL-2 and/or TNF. The reduction in Foxp3 expression by TNFR2-deficient Tregs was considerably greater than by WT Tregs, and more than 95% of initial Tregs derived from TNFR2$^{-/-}$ mice lost their Foxp3 expression (Fig 8D). Consequently, less than 10% of Foxp3-expressing cells in cLP were derived from TNFR2$^{-/-}$ Tregs which was markedly lower than those from WT mice ($p<0.0005$, Fig 8D). These data clearly show that TNFR2 per se is critical to sustain Foxp3 expression by Tregs in this model. In our experimental system, after transfer into Rag1$^{-/-}$ mice (CD45.2^{+}), TNFR2$^{-/-}$ Tregs isolated from CD45.2^{+} mice were outnumbered by WT Tregs derived from either CD45.2^{+} mice (Fig 7) or CD45.1^{+} mice (Fig 8), mitigating the potential impact of different congenic marker expressed by WT Tregs and TNFR2$^{-/-}$ Tregs.

Discussion

Our data presented in this study clearly show that TNFR2 plays a critical role in sustaining Foxp3 expression, and consequently maintaining the phenotypic and functional stability of Tregs. Therefore, at least in the inflammatory environment, the TNF/TNFR2 pathway is critical for the stabilization of Treg pool that is required to restrain the magnitude and length of an inflammatory immune response and to avoid harmful damage to self tissues.

A previous study reported that, in the same mouse colitis model, the accumulation and Foxp3 expression by TNFR2$^{-/-}$ Tregs in cLP were not changed when cells were harvested two weeks after transfer (22). Since the development of colitis in this model usually starts 5 weeks after transfer of naïve cells, we examined accumulation of Tregs and their Foxp3 expression when full-fledged colitis was developed which occurred typically 8 weeks after cell transfer. In our transfer experiments, both WT and TNFR2$^{-/-}$ Tregs were flow-sorted CD4^{+}CD25^{+}CD45RB^{lo} cells and contained similar high levels of Foxp3^{+} cells (>95%). The expansion of contaminating Foxp3^{-} Teff cells present in the initial Treg population may partially account for the reduction in Foxp3-expressing cells after transfer into Rag1$^{-/-}$ mice. However, the impact of this contamination population is very limited since it has been shown that the transferred Foxp3^{+} and Foxp3^{-} T cells were replicating similarly in the

lymphopenic mouse. There was no marked outgrowth of an intentionally added 3% Foxp3$^-$ Teffs in Tregs after transfer (36, 37). Further, TNFR2 is well known for its co-stimulatory effect on Teffs (38), thus it is likely that TNFR2$^{-/-}$ Teffs present in TNFR2$^{-/-}$ Tregs are less proliferative than WT Teffs.

IL-2 produced by Teffs has been proposed to be a paracrine factor to maintain Foxp3 expression and phenotypic as well as functional stability of Tregs (39). There are number of observations challenging the dispensable role of IL-2 on Tregs. 1) Only ~50% reduction of Foxp3$^+$ Tregs in CD4 cells was found in the thymus and peripheral lymphoid tissues of Il2$^{-/-}$ mice or Il2ra$^{-/-}$ mice, and residual Tregs had normal suppressive function (40). 2) A recent study found that the stability of transferred Tregs in the lymphopenic mouse was maintained by Teff cells independent of IL-2, since the administration of IL-2 did not promote the maintenance of Foxp3 expression by Tregs (37). 3) The stimulatory effect of Teffs on Tregs in vivo was not abrogated by neutralization of IL-2, and IL-2 deficient Teffs still had the capacity to stimulate the activation of Tregs (23). TGFβ is able to induce Foxp3 expression (41) and thus may play a role in the persistent expression of Foxp3 on Tregs. Nevertheless, conditional deletion of TGFβ receptor I (TβRI) in T cells also only delayed the appearance of Foxp3+ Tregs in neonatal mouse thymus, however, beginning 1 week after birth, the same TβRI-mutant mice showed accelerated expansion of thymic Tregs (42). The proportion of Tregs in mice devoid of TNFR2 or its ligand reduced by ~50% in both thymus and periphery. These data suggest that multiple factors, including TNF-TNFR2 signaling, contribute to the thymic generation and peripheral homeostasis of Tregs. Recently, Cuss and Green reported that thymic Tregs actually were heterogeneic and contained resident Tregs and newly developed Tregs. They proposed that resident Tregs were IL-2 dependent for their homeostasis, whereas newly developed Tregs were not (43). Further study is warranted to determine which subset of thymic Tregs is defective in mouse strains deficient in TNFR2 or its ligands, as well as in Il2$^{-/-}$ or Il2ra$^{-/-}$ and TβRI$^{-/-}$ mice.

In conclusion, TNFR2 is a key factor in maintaining sustained Foxp3 expression and function of Tregs contributing to immune regulation in the inflammatory environment, which may explain why anti-TNF therapy fails or even at times exacerbates some autoimmune disorders (44). This should be taken into account when designing future therapy of autoimmunity by using TNF inhibitors.

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Chapter 7

Expression of co-stimulatory TNFR2 induces resistance of $CD4^+FoxP3^-$ conventional T cells to suppression by $CD4^+FoxP3^+$ regulatory T cells

Xin Chen, Ryoko Hamano, Jeffrey J. Subleski, Arthur A. Hurwitz, O.M.Zack Howard and Joost J. Oppenheim

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Abstract

Our previous study showed that TNFR2 is preferentially expressed by $CD4^+FoxP3^+$ regulatory T (Treg) cells and expression of this receptor identified maximally suppressive Treg cells. TNFR2 is also expressed by a small fraction of $CD4^+FoxP3^-$ conventional T (Tconv) cells in normal mice and its expression is up-regulated by T cell activation. This raises questions concerning the role of TNFR2 signaling in the function of Tconv cells. In this study, by using FoxP3/gfp KI mice, we showed that TNFR2 signaling did not induce $FoxP3^-$ CD4 cells to become suppressive. Ki-67, a marker of proliferation, was concomitantly expressed with TNFR2 by CD4 cells, independent of FoxP3 expression, in both normal mice and Lewis Lung Carcinoma (LLC)-bearing mice. TNFR2 is associated with greater suppressive functions when expressed by Treg cells and is associated with greater resistance to suppression when expressed by Tconv cells. In mice bearing 4T1 breast tumor or LLC, intratumoral Tconv cells expressing elevated levels of TNFR2 acquired the capacity to resist suppression by LN-derived Treg cells. However, they remained susceptible to inhibition by more suppressive tumor infiltrating Treg cells which expressed higher levels of TNFR2. Our data indicate that TNFR2 also co-stimulates Tconv cells. However, intratumoral Treg cells expressing more TNFR2 are able to overcome the greater resistance to suppression of intratumoral Tconv cells, resulting in a dominant immunosuppressive tumor environment.

Introduction

Naturally arising $CD4^+FoxP3^+$ regulatory T (Treg) cells, comprising ~10% of peripheral $CD4^+$ T cells, play an important role in preventing immunopathology by suppressing immune responses to autoantigens (1). However, they also attenuate natural and induced immune responses against tumor antigens and therefore represent a mechanism by which tumors evade immune destruction (2). It has been reported that the intratumoral removal or inactivation of Treg cells enhanced anti-tumor immunity and resulted in eradicating advanced tumors (3-4), suggesting that the suppression of anti-tumor immunity by Treg cells occurs predominantly at the tumor site. Intratumoral $CD4^+$ effector T cells are crucial helpers of cytotoxic CD8 cell-mediated antitumor effect (5-6). Therefore, the balance maintained by Treg and Tconv subsets of CD4 cells in the tumor microenvironment is likely to determine whether the host immune responses evoked by tumor antigen results in tolerance or anti-tumor responses.

TNFR2 has co-stimulatory function and enhances the responses of lymphocyte to TCR-mediated signaling (7-9). Our previous study revealed that TNFR2 identifies a subset of Treg cells with maximally suppressive capacity in normal mouse lymphoid tissue. Furthermore, tumor infiltrating Treg cells were characterized by a high level of TNFR2 expression and markedly enhanced suppressive activity (10), presumably activated by TNF, a major proinflammatory mediator in the tumor microenvironment (11). We and other have reported that, in both human and mouse resting CD4 cells, TNFR2 is predominantly expressed by Treg cells (10, 12-13). Although only a minor fraction of normal mouse $CD4^+CD25^-$ Tconv cells express TNFR2, their expression of this receptor was also up-regulated in the tumor environment (10) or upon activation by in vitro TCR stimulation (14). Thus, endogenous TNF present in the tumor environment may also co-stimulate Tconv cells through TNFR2. It is crucial to further define the effect of the intratumoral co-stimulatory TNF-TNFR2 pathway on Tconv cells and Treg cells, since both TNF and anti-TNF have been used to treat cancer (11).

We report our novel observations that intratumoral Tconv cells with enhanced TNFR2 expression exhibited an activated phenotype and acquired the capacity to completely resist the suppressive effects of lymph node (LN)-derived Treg cells. However, intratumoral Treg cells expressed much higher level of TNFR2 and retained the capacity to markedly suppress intratumoral Tconv cells and consequently resulted in a dominant immunosuppressive tumor environment.

Material and methods

Mice, cells and reagents. Female wild type 8 to 12 wk old C57BL/6, Balb/c mice were provided by the Animal Production Area of the NCI (Frederick, MD). FoxP3/gfp KI mice were kindly provided by Dr. Yasmine Belkaid at Laboratory of Parasitic Diseases, NIAID, NIH, and maintained in the Animal Production Area of the NCI (Frederick, MD). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.). C57BL/6-derived Lewis lung carcinoma (LLC) cell line and Balb/c-derived 4T1 breast tumor cell line were obtained from ATCC. Antibodies (Abs) purchased from BD Biosciences (San Diego, CA) consisted of anti-CD3 (145-2C11), CD4 (GK1.5), anti-CD25 (PC61), CD45RB (16A), CD62L (MEL-14), CD44 (IM4), CD69 (H1.2F3), CD103 (M290), TNFR2 (TR75-89), CTLA4 (UC10-4F10-11), CD16/CD32 (2.4G2) and Ki-67 (B56). Functional grade purified anti-mouse CD3e Ab (eBio500A2), Foxp3 Staining Set (FJK-16s) and GITR (DTA-1) Abs were purchased from eBioscience (San Diego, CA). Anti-mouse CD120b (TR75-89) Ab was from AbD SeroTec (Kidlington, Oxford, UK).

Mouse tumor inoculation and separation of tumor infiltrating lymphocytes (TILs). LLC tumor cells were inoculated subcutaneously in the right flank of C57BL/6 mice in a single cell suspension of 500,000 cells in 0.1 ml PBS per mouse. 4T1 tumor cells were injected into both left and right mammary fat pads (thoracic #2 mammary glands) of recipient Balb/c mice in single cells suspension with 100,000 cells in 0.1 ml PBS per mouse. After two weeks, tumors were excised, minced and digested in RPMI 1640 supplemented with 1 mg/ml collagenase IV and 0.1 mg/ml DNase I. The fragments were pushed through a 70-µm pore size cell strainer to create a single-cell suspension. After density centrifugation of 40%-80% percoll, lymphocytes enriched interphase was collected, washed and stained with antibody for flow cytometry purification of TIL Treg cells. In some experiments, LLC tumor bearing mice were sacrificed at day 1, 5, 10 and 15 after inoculation of tumor cells, and Tregs present in the lymphoid tissues and TILs were analyzed.

Cell purification. CD4 subsets were purified and pooled from spleen and lymph nodes (inguinal, axillary and mesenteric regions) or TILs using Cytomation MoFlo cytometer (Fort Collins, CO), yielding a purity of ~98% for both subsets. T-depleted spleen cells were used as APCs and were prepared by depletion of $CD90^+$ cells with anti-mouse CD90 MicroBead and LD column (Miltenyi Biotec Inc.). APC were irradiated with 3000 R.

In vitro T cell proliferation assay. $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells from tumor bearing mouse LNs or tumor infiltrating lymphocytes, or $CD4^+FoxP3/gfp^+$ and $CD4^+FoxP3/gfp^-$ cells, with or without TNFR2 expression, were flow-sorted. The sorted cells were cultured alone or co-cultured at the desired ratio in a U-bottom 96-well plate in a medium with 2×10^5 APCs/well plus 0.5 μg/ml of soluble anti CD3 Ab. Cells were pulsed with 1 μCi [^{3}H]thymidine (Perkin Elmer Life Sciences, Boston, MA) per well for the last 6 h of the culture period. In some experiments, $CD4^+FoxP3/gfp^+$ Tregs were co-cultured with freshly isolated $CD4^+FoxP3/gfp^-TNFR2^-$ Tconv cells, or added to $CD4^+FoxP3/gfp^-TNFR2^-$ Tconv cells which were pre-activated with APCs (2×10^5 APCs/well) and anti-CD3 (0.5 μg/ml) for 24 hours. In some experiments, flow-sorted $CD4^+CD25^-TNFR2^-$ Tconv cells from normal Balb/c mice were cultured with 2-fold APCs and anti-CD3 (0.5 μg/ml) for 72 h. The cells were flow-sorted into $TNFR2^+$ and $TNFR2^-$ fractions. The re-sorted cells (2.5×10^4 cells/well) were cultured alone or co-cultured with freshly isolated Tregs at a desired ratio. The cells were stimulated with APCs (2×10^5 APCs/well) and anti-CD3 Ab (0.5 μg/ml). After incubation for 72 h, the proliferation was determined by [3] thymidine incorporation assay. IFNγ levels in the cell culture medium were determined by SearchLight Mouse Cytokine Array (Pierce Biotechnology, Woburn, MA).

Flow Cytometry. After blocking FcR, cells were incubated with appropriately diluted antibodies. Acquisition was performed using a FACSort or a SLRII (BD Biosciences, Mountain View, CA) and data analysis was conducted using FlowJo software (Tree Star Inc., Ashland, OR).

Statistical analysis. Comparison of data was analyzed by two-tailed Student's *t* test using Graphpad Prism 4.0.

Results

Both TNFR2-expressing Treg cells and Tconv cells in normal mice are more proliferative

To date, FoxP3 remains the best marker to identify mouse Treg cells (15). Some of the $FoxP3^+$ Treg cells are $CD25^-$ even in the normal mice (10). We therefore further defined the relationship of the expression of TNFR2 to FoxP3 expression by CD4 cells in normal C57BL/6 mice and FoxP3/gfp KI mice. Our current data corroborate our previous observations showing the greater TNFR2 expression on $CD4^+CD25^+$ Treg cells (10, 14). As shown in Figure 1A, TNFR2 was preferentially expressed by 47% of unstimulated $FoxP3^+$ splenic CD4 cells, while only ~4% of resting $FoxP3^-$ CD4 cells expressed low levels of TNFR2. Treg cells, which can be unequivocally identified by GFP expression in FoxP3/gfp

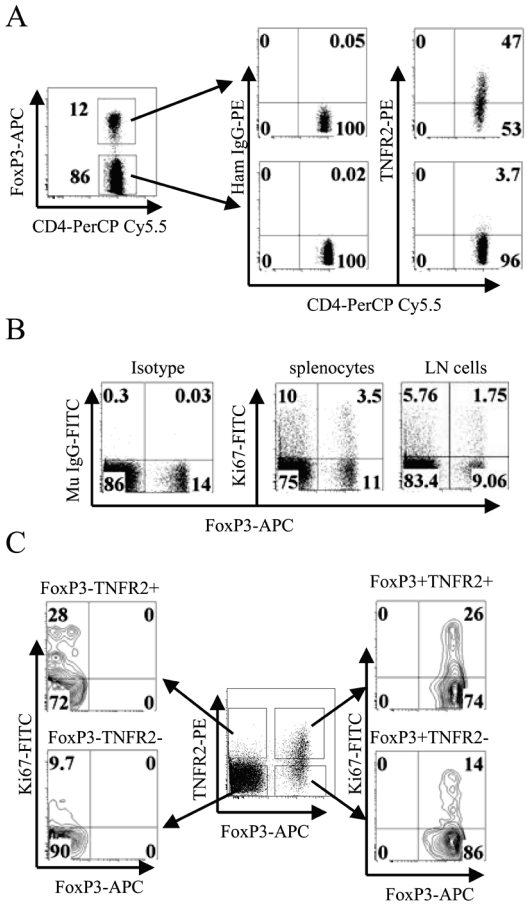

Figure 1. **Concomitant expression of TNFR2 and Ki-67 by CD4 subsets from normal mice.** (A) Spleen cells from normal C57BL/6 mice were stained with anti CD4, TNFR2 and FoxP3 Abs. The expression of TNFR2 by $CD4^+FoxP3^+$ and $CD4^+FoxP3^-$ cells was analyzed by FACS. (B) Spleen cells and axillary and inguinal LN cells from normal C57BL/6 mice were stained with anti CD4, Ki-67 and FoxP3 Abs. The expression of Ki-67 and FoxP3 was analyzed with FACS by gating on CD4. (C) Spleen cells from normal C57BL/6 mice were stained with anti CD4, TNFR2, FoxP3 and Ki-67 Abs. The expression of Ki-67 by $FoxP3^-TNFR2^+$, $FoxP3^-TNFR2^-$, $FoxP3^+TNFR2^-$ and $FoxP3^+TNFR2^+$ subsets was analyzed with FACS by gating on CD4 cells. Numbers indicate the percentage of cells in the respective quadrant. Data shown are representatives of at least three separate experiments with similar results.

KI mice, showed a similar pattern of TNFR2 expression on CD4 subsets from wild type mice: 45.9% of $FoxP3/gfp^+$ cells were TNFR2 expressing cells, and only 6.4% of $FoxP3/gfp^-$ CD4 cells expressed TNFR2 (data not shown).

TNFR2 has co-stimulatory functions which markedly enhances the activation of lymphocytes by TCR signals (7-9). This led us to hypothesize that TNFR2-expressing Treg cells and Tconv cells are likely to be more proliferative. We therefore assayed for Ki-67 expression because Ki-67 is exclusively expressed by proliferating cells (16). Furthermore, $Ki\text{-}67^{hi}$ CD4 cells are reported to express an increased level of TNFR2 (17). Consistent with this observation, normal C57BL/6 mouse splenic $CD4^+FoxP3^+$ cells with higher expression of TNFR2 comprised 2-fold higher $Ki\text{-}67^+$ cells (24%) than $CD4^+FoxP3^-$ cells (12%). In LNs from inguinal and axillary regions, 16.2% of $CD4^+FoxP3^+$ cells were $Ki\text{-}67^+$ cells, compared with 6.5% of $CD4^+FoxP3^-$ cells, although the overall Ki-67 levels were lower

than in splenocytes (Fig 1B). The Ki-67$^+$ cells in the FoxP3$^+$ splenic CD4 subset were largely present in the TNFR2$^+$ subset (26%). Although a low level of Ki-67 was expressed in the population of FoxP3$^-$ CD4 cells, 28% of the TNFR2-expressing cells in this subset also were Ki-67$^+$ cells. In contrast, only 14% and 9.7% of Ki-67$^+$ cells were present in TNFR2 negative FoxP3$^+$ and FoxP3$^-$ CD4 cells respectively (Fig 1C). Thus, Tregs and Tconv cells in normal mouse lymphoid tissues exhibited concomitant expression of TNFR2 and Ki-67.

TNFR2$^+$ Treg cells potently inhibited proliferation of more resistant TNFR2$^+$ Tconv cells

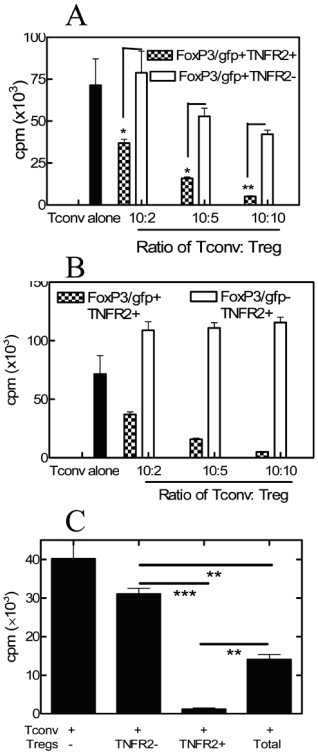

Figure 2. Suppressive activity of CD4 subsets from FoxP3/gfp KI mice. Flow-sorted CD4$^+$FoxP3/gfp$^-$TNFR2$^-$ cells (Tconv cells), 5×10^4 cells/well, were cultured alone or co-cultured with flow-sorted CD4$^+$FoxP3/gfp$^+$TNFR2$^+$ or CD4$^+$FoxP3/gfp$^+$TNFR2$^-$ cells (A), or CD4$^+$FoxP3/gfp$^+$TNFR2$^+$ or CD4$^+$FoxP3/gfp$^-$TNFR2$^+$ cells (B) from FoxP3/gfp KI mice, at a ratio of 10:2, 10:5 and 10:10 (Tconv cells: Treg cells). In other experiment, 2.5×10^4 cells/well of CD4$^+$FoxP3/gfp$^-$TNFR2$^-$ cells (Tconv) were cultured alone or co-cultured at ratio of 1:1 with CD4$^+$FoxP3/gfp$^+$TNFR2$^+$ (TNFR2$^+$ Tregs), or CD4$^+$FoxP3/gfp$^+$TNFR2$^-$ cells (TNFR2$^-$ Tregs), or unfractionated CD4$^+$FoxP3/gfp$^+$ cells (Total Tregs) (C). The cells were stimulated with APCs (2×10^5 cells/well) and anti-CD3 (0.5 μg/ml) for 72 hours. Proliferation was measured by [3H]thymidine incorporation. Comparison of proliferation of co-cultures including FoxP3/gfp$^+$TNFR2$^+$ with co-cultures including FoxP3/gfp$^+$TNFR2$^-$ cells in (A), * $p<0.05$; ** $p<0.01$. Comparison of proliferation of indicated co-cultures in (C), ** $p<0.01$, ***$p<0.001$. Data (mean ± SD, N=3) shown are representatives of at least 3 separate experiments with similar results.

We next sought to determine whether TNFR2$^+$ Tconv cells may be more resistant to suppression by Treg cells, by taking the advantage of FoxP3/gfp KI mice which allows purification of viable FoxP3$^+$ Treg subsets based on GFP expression. First, we confirmed our previous observation that the suppressive capacity of TNFR2$^+$ Tregs were considerably greater than those of TNFR2$^-$ Tregs over various ratios of Tconv:Treg ($P<0.001$~0.05, Fig 2A, C) or of unfractionated Tregs (CD4$^+$FoxP3/gfp$^+$ cells) which usually consist of ~40% of TNFR2$^+$ cells ($p<0.01$, Fig 2C). In fact, unfractionated Tregs were more suppressive than

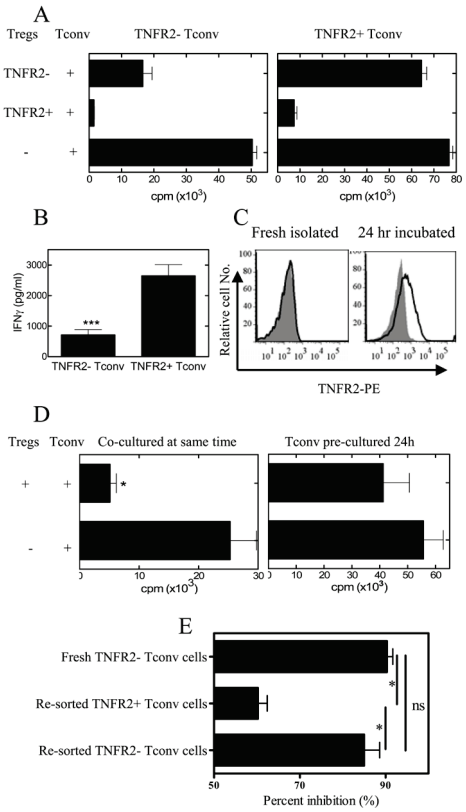

Figure 3. **TNFR2$^+$ Tconv cells are resistant to Treg-mediated inhibition**. TNFR2$^-$ Tconv cells (CD4$^+$FoxP3/gfp$^-$TNFR2$^-$) (left) or TNFR2$^+$ Tconv cells (CD4$^+$FoxP3/gfp$^-$TNFR2$^+$) (right), 5×10^4 cells/well, were cultured alone or co-cultured with TNFR2$^+$ Treg cells (CD4$^+$FoxP3/gfp$^+$TNFR2$^+$) or TNFR2$^-$ Treg cells (CD4$^+$FoxP3/gfp$^+$TNFR2$^-$) at a ratio of 1:1. The cells were stimulated with APCs (2×10^5 cells/well) and anti-CD3 (0.5 μg/ml) for 72 hours. Proliferation was measured by [3H]thymidine incorporation. (B) IFNγ levels in TNFR2$^-$ and TNFR2$^+$ Tconv cells which were cultured alone with same condition as in (A). Comparison of two gropus, *** $p<0.001$. (C) Flow-sorted CD4$^+$FoxP3/gfp$^+$TNFR2$^-$ Tconv cells, fresh isolated (left) or cultured with APCs (2×10^5 cells/well) and anti-CD3 (0.5 μg/ml) for 24 hours were stained with anti CD4 and TNFR2 Abs. Expression of TNFR2 was analyzed by gating on CD4 cells. (D) Flow-sorted CD4$^+$FoxP3/gfp$^+$TNFR2$^-$ Tconv cells, fresh isolated (left) or cultured with APCs (2×10^5 cells/well) and anti-CD3 (0.5 μg/ml) for 24 hours, were cultured alone or co-cultured at 1:1 ratio with flow-sorted Tregs. The cells were stimulated with APCs and anti CD3 Ab for 72 hours. Proliferation was measured by [3H]thymidine incorporation. Comparision of proliferation of Treg/Tconv co-cultures with that of Tconv culture alone, * $p<0.05$. (E) Flow-sorted CD4$^+$CD25$^-$TNFR2$^-$ Tconv cells from normal Balb/c mice were cultured with 2-fold APCs and anti CD3 Ab (0.5 μg/ml). After 72 h incubation, the cells were flow-sorted into TNFR2$^+$ and TNFR2$^-$ fractions. The re-sorted cells (2.5×10^4 cells/well) were cultured alone or co-cultured with freshly isolated CD4$^+$CD25$^+$ Tregs at a ratio of 2:1 (Teffs: Tregs). Freshly flow-sorted CD4$^+$CD25$^-$TNFR2$^-$ Tconv cells were used as a comparison. The cells were stimulated with APCs (2×10^5 cells/well) and anti-CD3 Ab (0.5 μg/ml) for 72 h, and proliferation was determined by [3]H thymidine incorporation assay. Percent inhibition (%) was shown. Comparison of two groups, * $p<0.05$. ns = not significant. Data in (A, B, D and E) stand for mean ± SD (N=3). Data shown are representatives of at least 3 separate experiments with similar results.

TNFR2$^-$ Tregs ($p<0.01$, Fig 2C). In contrast, TNFR2-expressing FoxP3/gfp$^-$ CD4 cells did not have any suppressive activity at all (Fig 2B), indicating that TNFR2 by itself was not a specific indicator of CD4 suppressive cells. Therefore, the suppressive activity of CD4$^+$CD25$^-$TNFR2$^+$ T cells present in normal C57BL/6 mice (10) was solely attributable to the more highly suppressive subset of TNFR2$^+$FoxP3$^+$ cells present in this CD4$^+$CD25$^-$ subpopulation.

Furthermore, TNFR2$^+$ Treg cells

potently inhibited proliferation of both $TNFR2^-$ and $TNFR2^+$ Tconv cells. In contrast, although $TNFR2^-$ Treg cells exerted a weaker but detectable inhibitory effect on $TNFR2^-$ Tconv cells, this subset of Treg cells had no suppressive activity when co-culture with $TNFR2^+$ Tconv cells (Fig 3A). Upon stimulation with APC and anti-CD3, these more resistant $TNFR2^+$ Tconv cells produced ~5-fold more INFγ than $TNFR2^-$ Tconv cells ($p<0.001$, Fig 3B), indicating that $TNFR2^+$ Tconv cells were more activated. Thus, TNFR2 expression on Tconv cells enhances their capacity to resist Treg-mediated suppression, however, they were still susceptible to suppression by TNFR2-expressing Treg cells.

This led us to hypothesize that induced expression of TNFR2 on Tconv cells would render them refractory to suppression by Tregs. To test this, we activated flow-sorted $CD4^+FoxP3/gfp^-TNFR2^-$ cells with APC and anti-CD3 for 24 hours and found their expression of TNFR2 to be markedly up-regulated (Fig 3C). Unfractionated Tregs ($CD4^+FoxP3/gfp^+$) lost their capacity to inhibit the proliferation of these pre-activated Tconv cells (Fig 3D, right). In contrast, unfractionated Tregs potently inhibited the proliferation of unactivated $TNFR2^-$ Tconv cells ($p<0.05$, Fig 3D, left).

We further investigated whether only TNFR2-expressing Tconv cells acquired the Treg-resistant capacity or all pre-activated Tconv cells developed this property. To provide a definite answer to this question, we re-sorted pre-activated Tconv cells based on their TNFR2 expression and studied their responses to Tregs. Since our previous study showed that Tconv cells from Balb/c mice were more susceptible to inhibition by Tregs, as compared with Tconv cells from C57BL/6 mice (18), and we used Tconv cells from this mouse strain to examine the induction of Treg-resistance. First, we isolated $CD4^+CD25^-TNFR2^-$ cells from LNs and spleens of normal Balb/c mice by FACS cell sorting and the cells were stimulated with APCs and anti-CD3 Ab. After three days culture, ~30% of cells became TNFR2-expressing cells, which confirmed our previous report (14). The cells were re-sorted into $TNFR2^+$ and $TNFR2^-$ fractions, and their responses to Tregs were assessed by co-culturing them with freshly isolated $CD4^+CD25^+$ Tregs from normal Balb/c mice. As shown in Fig 3E, re-sorted $TNFR2^+$ Tconv cells were markedly more resistant than re-sorted $TNFR2^-$ Tconv cells to suppression of proliferation by Tregs ($P<0.05$). Interestingly, re-sorted $TNFR2^-$ Tconv cells were as susceptible as freshly isolated $TNFR2^-$ Tconv cells to suppression by freshly isolated Tregs ($P>0.05$), indicating that the enhanced Treg-resistant capacity of activated Tconv cells was exclusively restricted to Tconv cells which were induced to express TNFR2. Thus, induced expression of TNFR2 on Tconv cells by TCR stimulation also enhanced their resistance to Tregs.

$TNFR2^+$ Tregs and Tconv cells in draining LNs are more proliferative than in non-dLNs in tumor-bearing mice

Our previous data showed that tumor infiltrating Tregs and Tconv cells expressed a high levels of TNFR2 (10), indicating they were highly proliferating cells. We therefore further compared Ki-67 expressed by $FoxP3^+$ and $FoxP3^-$ subsets of CD4 cells present in the spleen, draining LNs (dLN) and non-draining LNs (ndLN) of mice inoculated with LLC tumor cells for 15 days when the average tumor volume reached 301.6 mm^3 (N=6). As shown in Fig 4A, the proportion of Ki-67^+ cells in both FoxP3+ and $FoxP3^-$ CD4 subsets was not increased in the spleen and non-dLN of tumor-bearing mice. As expected, $FoxP3^+$ cells in the dLNs were more proliferative than in the ndLNs, as evidenced by their higher level of Ki-67^+ cells ($p<0.01$, Fig 4A, C). In contrast, the increase in Ki-67^+ cells in $FoxP3^-$ CD4 subsets present in the dLN did not differ from ndLN ($P>0.05$, Fig 4A, C). Thus, the proliferation of Treg cells in the tumor dLN is more robust than that of Tconv cells. Furthermore, 50~60% of $TNFR2^+FoxP3^+$ cells in dLNs were Ki-67^+, which was higher ($p<0.01$) than 20~30% of Ki-67^+ cells in ndLNs (Fig 4B, C). Similarly, the percentage of Ki-67^+ cells in $FoxP3^-TNFR2^+$ population, was higher ($p<0.05$) in the dLN (40~50%) than in ndLNs (20~30%, Fig 4B, C). In contrast, the proportion of Ki-67^+ cells in $FoxP3^+$ and $FoxP3^-$ subsets lacking TNFR2 expression each remained at an equally low level (<10%) in the dLNs and ndLNs ($P>0.05$, Fig 4B, C). We also examined the cellular numbers in the spleen, non-dLNs and dLNs. As shown in Table I, the total cellularity in the dLNs (34.8×10^6/mouse on average) was 4-fold greater than in the non-dLNs (8.5×10^6/mouse on average, $p<0.001$). The proportion of $FoxP3^+$ Tregs in the dLNs was actually more reduced ($p<0.05$) than in non-dLNs, this was presumably due to the increase in other cell type in the dLN, since the proportion of $FoxP3^+$ cells in the CD4 subset of dLN cells were not decreased (data not shown). It is relevant that, the number of $FoxP3^+$ Tregs in the dLNs (1.22×10^6/mouse on average) was almost 3-fold greater than in the non-dLNs (0.41×10^6/mouse on average, $p<0.001$). Consistent with the increase in Ki-67^+ cells in the dLN (Fig 4), the number of Ki-67^+ cells (0.37×10^6/mouse on average) was 4.6-fold greater than in the non-dLN (0.08×10^6/mouse on average, $p<0.001$). Thus, both the percentage and number of Ki-67^+ Tregs were increased in the dLN of tumor bearing mice.

After subcutaneous inoculation of LLC tumor cells into syngeneic C57BL/6 mice, the tumor developed over time and the average tumor weight was 25.8±7.3 mg, 88.1±38.0 mg

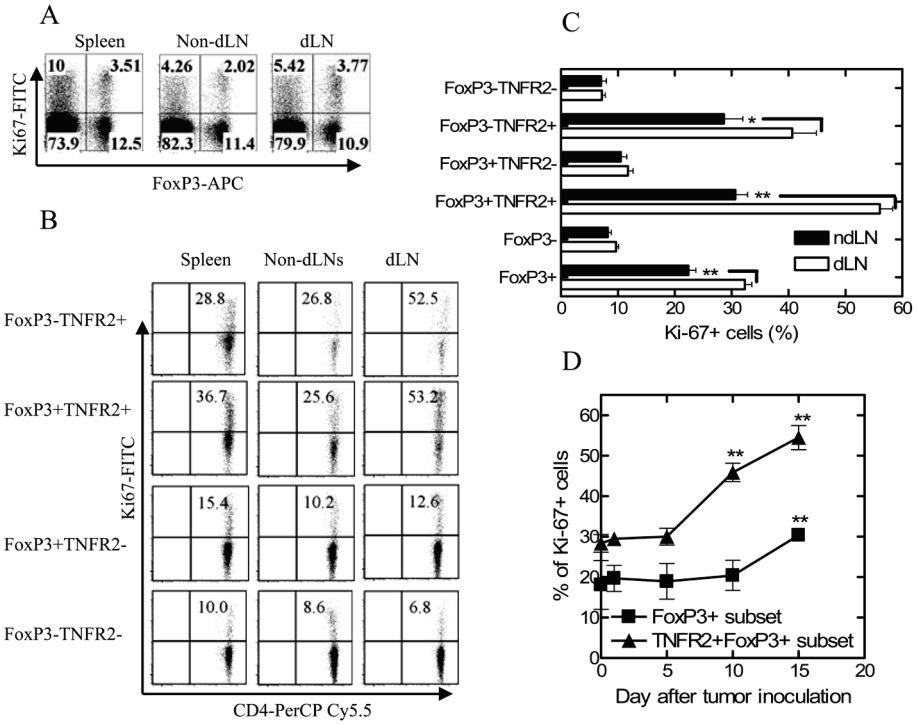

Figure 4. **Expression of Ki-67 by CD4 subsets in the lymphoid organs of tumor bearing mice**. Spleen cells, or non-draining LNs (non-dLNs, left side inguinal and axillary LNs), or draining LNs (dLN, right side inguinal and axillary LNs) from mice inoculated with LLC tumor cells for 15 days were stained with anti CD45, CD4, FoxP3, Ki-67 and TNFR2 Abs. (A) Expression of Ki-67 by $FoxP3^-$ and $FoxP3^+$ cells were analyzed by gating on $CD45^+CD4^+$ cells. (B) Expression of Ki-67 by $FoxP3^-TNFR2^+$, $FoxP3^+TNFR2^+$, $FoxP3^+TNFR2^-$ and $FoxP3^-TNFR2^-$ CD4 subsets present in spleen, Non-dLNs and dLNs were analyzed by gating on respective populations. Data shown in (A~B) are representatives of at least three separate experiments with similar results. Numbers in the dot plots indicate the percentage of cells in the respective quadrant. (C) Comparison of Ki-67 expression by CD4 subset present in non-dLN and dLN of tumor-bearing mice, *$p<0.05$, **$p<0.01$. Data (means ± SD) shown in (C) are summarized from three separate experiments with similar results (N=9). (D) Kinetic expression of Ki-67 by $FoxP3^+$ Tregs and $TNFR2^+FoxP3^+$ Tregs present in dLNs of mice inoculated with LLC tumor cells for 0, 1, 5, 10 and 15 days. Compared with tumor free mice (day 0), ** $p<0.01$. Data (means ± SD) shown in (D) are summarized from two separate experiments with similar results (N=6).

and 267.4±92.3 mg on day 5, day 10 and day 15 after tumor inoculation (N=6), respectively. We further examined the kinetics of Ki-67$^+$ Tregs in the dLN over this period of tumor development. The proportion of Ki-67$^+$ cells present in the total FoxP3$^+$ Tregs was markedly increased beginning by day 15, and by day 10 in the TNFR2$^+$FoxP3$^+$ subset of Tregs, respectively ($p<0.01$, Fig 4D). Nevertheless, at the earlier stage of tumor development, e.g., day 5 of tumor inoculation, the proportion of Ki-67$^+$ Tregs present in the dLNs was not different from that in the LNs in the same region of tumor free mice (day 0, $p>0.05$).

Both TNFR2$^+$ Treg and Tconv subsets of tumor infiltrating lymphocytes (TILs) are more proliferative

To determine the relationship between TNFR2 expresssion and proliferation by Tconv cells and Tregs in TILs, we assessed Ki-67 and TNFR2 expression by FoxP3$^+$ and FoxP3$^-$ CD4 TILs in C57BL/6 mice inoculated with LLC tumor cells at 15 days, at a stage of full tumor development. As shown in Fig 5A, 88.1% of FoxP3$^+$ cells present in CD4 TILs were TNFR2$^+$ cells, while only 52.8% of splenic FoxP3$^+$ cells expressed TNFR2. TNFR2-expressing FoxP3$^-$ CD4 TILs were 40.3%, while only 7% of splenic FoxP3$^-$ Tconv cells were TNFR2$^+$. Similarly, 84.7% and 48.8% of FoxP3$^+$ and FoxP3$^-$ TIL CD4 cells expressed Ki-67, respectively. In comparison, 22.4% and 12% of FoxP3$^+$ and FoxP3$^-$ splenic CD4 cells were Ki-67-expressing cells (Fig 5B). Similar data were also observed in LLC-bearing FoxP3/gfp KI mice and 4T1 mammary tumor-bearing Balb/c mice (data not shown). These data clearly show that TNFR2 expression by both FoxP3$^+$ and FoxP3$^-$ CD4 cells in tumors

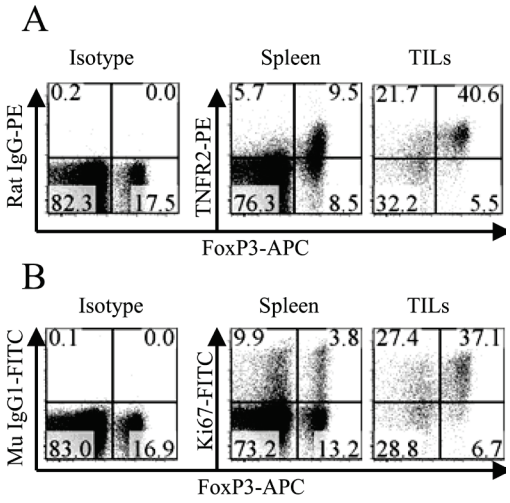

Figure 5. **Tumor infiltrating FoxP3$^+$ and FoxP3$^-$ subsets of CD4 cells up-regulated their TNFR2 expression and were actively replicatin**g. Spleen cells and TILs from C57BL/6 mice inoculated with LLC tumor cells for 15 days were stained with anti CD45, CD4, FoxP3 and Ki-67 Abs or isotype control Abs. (A) The expression of TNFR2 by FoxP3$^-$ cells and FoxP3$^+$ cells were analyzed by gating on CD45$^+$CD4$^+$ cells; (B) The expression of Ki-67 by FoxP3$^-$ cells and FoxP3$^+$ cells were analyzed by gating on CD45$^+$CD4$^+$ cells. Numbers indicate the percentage of cells in the respective quadrant. Data shown are representatives of at least three separate experiments with similar results.

was markedly increased, however, more of the FoxP3$^+$ Treg cells expressed TNFR2 and TNFR2 expression levels on Treg cells were much higher on a per cell basis than on FoxP3$^-$ Tconv cells, consistent with their Ki-67 expression.

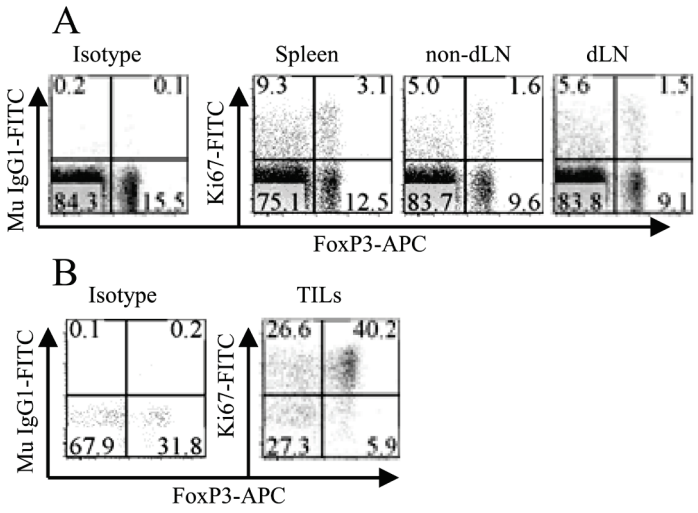

Figure 6. **The expression of Ki-67 by FoxP3$^+$ and FoxP3$^-$ subsets of CD4 cells in the lymphoid tissues and TILs from mice with early tumor development**. Spleen cells, non-dLN, dLN cells and TILs from C57BL/6 mice inoculated with LLC tumor cells for 5 days were stained with anti CD45, CD4, FoxP3 and Ki-67 Abs. The expression of Ki-67 by FoxP3$^-$ and FoxP3$^+$ cells were analyzed by gating on CD45$^+$CD4$^+$ cells. (A) lymphoid tissues; (B) TILs. Numbers indicate the percentage of cells in the respective quadrant. Data shown are representatives of two separate experiments with similar results.

We also determined the Ki-67 expression by TIL Tregs and Tconv cells at an earlier stage of day 5 after tumor cells inoculation. As shown in Fig 6A, at this stage, the proportion of Ki-67$^+$ cells in both FoxP3$^+$ and FoxP3$^-$ CD4 subsets present in the spleen, non-dLN and dLN was not increased, as compared with that in tumor free mouse (Fig 6A). Nevertheless, the proportion of FoxP3$^+$ cells in CD4 TILs was greater than 40%. Furthermore, 49.4% of FoxP3$^-$ and 87.2% of FoxP3$^+$ CD4 TILs expressed Ki-67 (Fig 6B). Thus, there was a ~2-fold greater number of Ki-67$^+$ cells in the FoxP3+ subset of TILs than in the FoxP3$^-$ subset of TILs at both day 5 and day 15 of tumor cell injection, suggesting that Treg cells in the tumor are more replicative and have a higher turnover than Tconv cells.

Tumor infiltrating Treg cells as well as Tconv cells have an activated phenotype

The enhanced expression of TNFR2 and Ki-67 by tumor infiltrating Treg cells and Tconv cells suggests they might have an activated phenotype. Isolation of Treg cells in wild type

mice is usually based on CD25 expression. We therefore compared the phenotype of 4T1 tumor infiltrating $CD4^+CD25^+$ cells and $CD4^+CD25^-$ cells from mice inoculated with tumor cells for 15 days. $CD4^+CD25^+$ cells in tumor-free Balb/c mouse LNs consisted of 93% of $FoxP3^+$ cells, while only ~2% of $CD4^+CD25^-$ cells expressed FoxP3 (Fig 7A). 90% of $CD4^+CD25^+$ TILs expressed FoxP3. In comparison, 10% of $CD4^+CD25^-$ TILs were $FoxP3^+$ (Fig 7A). Thus in 4T1 tumor, proportion of $FoxP3^+$ cells in $CD4^+CD25^-$ population was

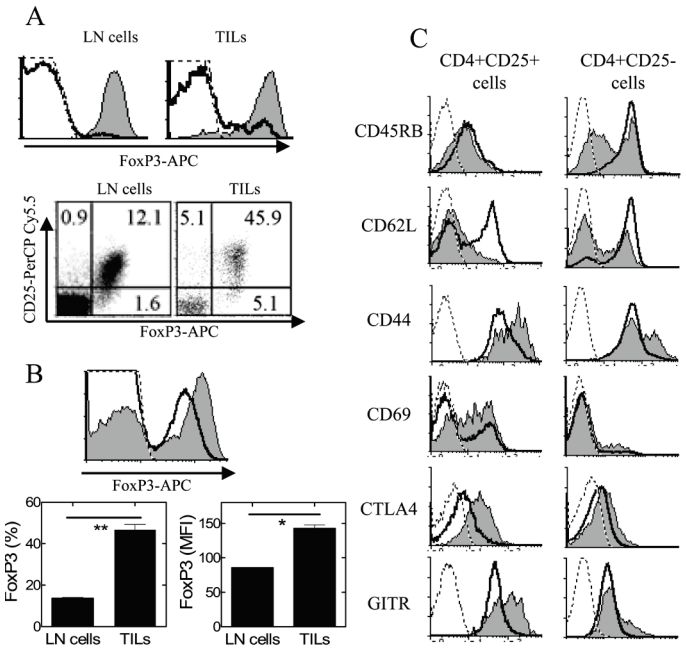

Figure 7. **Phenotype of $CD25^+$ and $CD25^-$ subsets of tumor infiltrating CD4 cells from 4T1 tumor bearing mice.** (A) LN cells from normal Balb/c mice or 4T1 tumor TILs were stained with anti CD45, CD4, CD25 and FoxP3 Abs. The expression of FoxP3 was analyzed by FACS, gating on $CD4^+CD25^+$ cells (grey histogram) or gating on $CD4^+CD25^-$ cells (solid line histogram, upper panel). The relationship of CD25 and FoxP3 expression on CD4 cells present in the control LN cells and in the TILs was shown in the dot plot (lower panel). (B) Expression of FoxP3 by total CD4 cells from tumor free Balb/c mouse LN cells (solid line histogram) or by tumor infiltrating CD4 cells from 4T1 tumor bearing mice (grey histogram) was analyzed by FACS, gating on $CD4^+$ cells (upper). The percentage (%) and MFI of $FoxP3^+$ cells in the CD4 population present in control LNs and in the TILs were shown in the lower panel. Data shown are Means ± SD (N=3). Comparison of two groups, *$p<0.05$, ** $p<0.01$. (C) Splenocytes from tumor free Balb/c mice (solid line histogram) and TILs from 4T1 tumor bearing mice (grey histogram) were stained with anti CD45, CD4, CD25 and phenotypic markers (CD45RB, CD62L, CD44, CD69, CTLA-4 and GITR). Expression of phenotypic marker was analyzed by FACS, gating on $CD4^+CD25^+$ cells (left panel) or $CD4^+CD25^-$ cells (right panel). Isotype controls were shown by dashed line histogram. Data shown are representatives of three separate experiments with similar results.

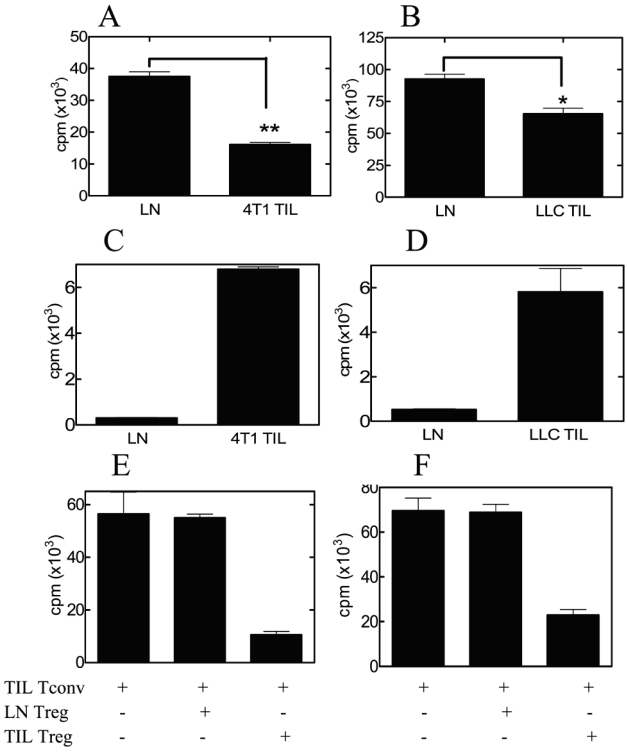

Figure 8. **Tumor infiltrating Treg cells potently inhibit tumor infiltrating Tconv cells**. (A~B) Proliferative responses of Tconv cells: FACS-sorted $CD4^+CD25^-$ cells from LNs of tumor free or from TILs of 4T1 tumor bearing Balb/c mice (A), or from LNs of tumor free or from TILs of LLC-bearing C57BL/6 mice (B) were cultured at 2.5×10^4 cells/well. The cells were stimulated with APCs (2×10^5 cells/well) and anti-CD3 for 72 h. Proliferation was measured by [3]thymidine incorporation. (C~D) Proliferative responses of Treg cells: FACS-sorted $CD4^+CD25^+$ cells from LNs of tumor free or from TILs of 4T1 tumor bearing Balb/c mice (C), or from LNs of tumor free or LLC-bearing C57BL/6 mice (D) were cultured at 2.5×10^4 cells/well. The cells were stimulated with APCs (2×10^5 cells/well) and anti-CD3 for 72 h. Proliferation was measured by [3]thymidine incorporation. (E~F) Suppressive activity of Treg cells: flow-sorted $CD4^+CD25^-$ cells (2.5×10^4 cells/well) from TILs of 4T1 tumor bearing Balb/c mice were cultured alone or co-cultured with same number of $CD4^+CD25^+$ cells from LNs of tumor free or TILs of 4T1 tumor-bearing Balb/c mice (E), or $CD4^+CD25^-$ cells (2.5×10^4 cells/well) from TILs of LLC tumor bearing C57BL/6 mice were cultured alone or co-cultured with same number of $CD4^+CD25^+$ cells from LNs of tumor free or TILs of LLC tumor-bearing C57BL/6 mice (F). The cells were stimulated with APCs (2×10^5 cells/well) and anti-CD3 for 72 h. Proliferation was measured by [3]thymidine incorporation. Data (mean ± SD, N=3) shown are representatives of at least three separate experiments with similar results.

increased, as compared with cells from LNs. The proportion of $FoxP3^+$ cells in CD4 TILs and the intensity of FoxP3 expression by TIL CD4 cells was greater than that of LN CD4 cells from tumor free mice ($p<0.01$ and $p<0.05$, respectively, Fig 7B), this presumably resulted from stimulation by TNF, which has the capacity to increase the expression level of FoxP3 by Treg cells on a per cell basis (14). Our results differ from those in a previous report showed that Treg cells in 4T1 tumor at a later stage by day 28 after tumor inoculation expressed a lower level of FoxP3 expression (5), the reason for this discrepancy will be addressed in a future study.

Consistent with our previous results that $TNFR2^+$ Treg cells in the normal mouse had an activated phenotype, $CD4^+CD25^+$ TILs also exhibited a lower level of CD62L, higher levels of CD44, CD69, CTLA-4 and GITR, as compared with LN $CD4^+CD25^+$ cells from tumor free mice. Moreover, TIL $CD4^+CD25^-$ T cells also exhibited an activated phenotype, as evidenced by lower levels of CD45RB, CD62L and increased levels of CD44, CTLA-4 and GITR than $CD4^+CD25^-$ T cells from tumor free mouse LNs (Fig 7C). Therefore, tumor infiltrating Tconv cells are also activated at the tumor site.

Tumor infiltrating Tconv cells are refractory to suppression by LN Tregs, but remain susceptible to inhibition by TIL Tregs

Next we compared the TCR dependent proliferative responses of tumor infiltrating Treg cells and Tconv cells. $CD4^+CD25^-$ TILs from both 4T1 tumor or LLC tumor exhibited lower proliferative responses upon APC/anti-CD3 stimulation than $CD4^+CD25^-$ T cells from LNs ($p<0.01$~0.05, Fig 8A~B). In contrast, $CD4^+CD25^+$ TILs from both 4T1 and LLC tumors exhibited an greater proliferative response to APC/anti-CD3 stimulation than the hyporesponssive $CD4^+CD25^+$ cells from tumor free mouse LNs (Fig 8C~D). Interestingly, although $CD4^+CD25^-$ TILs from both 4T1 and LLC tumors exhibited attenuated proliferative responses to TCR stimulation, unlike $CD4^+CD25^-$ responder cells from tumor free Balb/c and C57BL/6 mice (18), they resisted suppression by $CD4^+CD25^+$ cells from tumor free mouse LNs at a ratio of 1:1 (Figure 8E~F). Thus, consistent with their elevated levels of TNFR2 and Ki-67, TIL Tconv cells were more resistant to suppression by LN-derived Tregs. Nevertheless, when co-cultured at 1:1 ratio, $CD4^+CD25^+$ TILs from both 4T1 and LLC tumors exhibited a potent suppressive effect on the proliferation of tumor infiltrating $CD4^+CD25^-$ cells (Fig 8E~F). Therefore, in the tumor environment, the functional capabilities of both Treg cells and Tconv cells were altered, but Treg-mediated suppression still remained dominant over the more resistant Tconv TIL cells.

Discussion

The failure of Treg cells to proliferate after TCR stimulation in vitro initially led to their classification as naturally anergic cells. Our data show that FoxP3$^+$ CD4 cells actually expressed higher frequency of Ki-67$^+$ cells than FoxP3$^-$ CD4 cells from normal mice, suggesting that even in the steady-state, FoxP3$^+$ CD4 cells are undergoing more robust replication and have higher turnover than FoxP3$^-$ CD4 cells. Our data are therefore consistent with a previous report that a subset of Tregs in normal mice proliferated even in the steady state, based on CFSE-dilution and BrdU incorporation assays (19). Furthermore, the expression of Ki-67 correlated directly with TNFR2 expression, which is in agreement with our current understanding of TNFR2 as a co-stimulator of the proliferative response of lymphocytes to TCR stimulation (20-22). The cell source of TNFR2 ligands which potentially contribute to the homeostasis of Treg pool in the steady-state remain to be defined.

The basis for the expansion of Treg cells at the tumor site remains elusive. Three mechanisms have been proposed to account for the accumulation of Treg cells in the tumor mass: 1) the tumor microenvironment actively recruits natural Treg cells by producing chemokine ligands which interact with chemokine receptor expressed by Treg cells (23); 2) naïve CD4 cells can be converted into tumor-specific FoxP3$^+$ Treg cells by TGFβ produced by tumor cells (24) or alternatively by PD-L1 expressed by DCs in the tumor (25); 3) the tumor microenvironment stimulates the proliferative expansion of infiltrated Treg cells (26). It has been proposed that TGFβ produced by DCs exhibiting an immature phenotype could stimulate the proliferation of Treg cells in tumor (27). However, although known to induce *de novo* differentiation of naïve CD4 cells into Treg cells (28), TGFβ is actually a potent suppressor of proliferation of T cells (29). We have observed that TGFβ also inhibits TNF-induced proliferation of Treg cells in vitro (unpublished data). At day 5 after tumor inoculation, <20% of the FoxP3$^+$ Tregs in the dLN were proliferating (Fig 4D and Fig 6), while more than 80% of TIL FoxP3$^+$ Treg cells are Ki-67$^+$ (Fig 6). These data indicate that *in situ* proliferative expansion contribute substantially to the accumulation of Tregs in the tumor. TNF is a key mediator of cancer-associated inflammation, produced either by tumor cells and/or by infiltrating leukocytes (11) and has the capacity to activate Treg cells (14). IL-2 is known to be critical to the survival and proliferation of Tregs (30). However, the expression of IL-2 in the tumor tissue is low or absent (31-32), presumably due to the hypoxic tumor environment since IL-2 transcription is exquisitely sensitive to changes in oxygen tension (33), thus argue against the likelihood of a major role of IL-2 in the expansion of Tregs in the tumor. Although low levels of IL-2 are needed for survival of

Tregs, the TNF enriched tumor microenvironment likely induces the proliferative expansion of $TNFR2^+$ Treg cells through TNF-TNFR2 interaction.

A small fraction of Tconv cells constitutively express TNFR2 even in the steady-state (Fig 1) and TNFR2-expressing Tconv cells are increased in the tumor (Fig 5-6). It has been shown that memory Tconv cells are refractory to Treg-mediated inhibition (34) and that interaction of TNFR2 with its ligands plays a role in the maintenance of T cell memory (35). Whether $TNFR2^+$ Tconv cells in normal or tumor bearing mice actually represent memory cells merits future study. Furthermore, activated Tconv cells express more TNFR2 [(14) and Fig 3C]. Such activated $TNFR2^+$ Tconv cells were refractory to Treg-mediated suppression. Indeed, $CD4^+FoxP3/gfp^-TNFR2^-$ cells pre-activated in vitro with APCs/anti-CD3 also acquired the capacity to resist suppression by Tregs, accompanied by up-regulation of TNFR2 (Fig 3C~D), indicating that proliferating Tconv cells become more resistant than resting Tconv cells to Tregs. Importantly, induction of Treg-resistance by TCR-stimulation was restricted to the Tconv cells which were induced to express TNFR2 (Fig 3E), underscoring the role of TNFR2 in the Treg-resistance. TNFR2 has been shown to promote TCR-mediated cellular activation (7-9). A recent study reported that the activation of TNFR2, but not TNFR1, on human T cells resulted in p100 processing and induced the activation of alternative NFκB pathway (36). Thus, expression of TNFR2 empowers Tconv cells to respond to the stimulation of its ligands, and consequently enhances their resistance to the inhibition by Tregs.

It was reported that Tconv cells present in the 4T1 tumor with an activated phenotype were hyporesponsive to ex vivo TCR stimulation (5). Similarly, we found that tumor infiltrating $CD4^+CD25^-$ cells exhibited an activated phenotype (Fig 7) and comprised a higher proportion of $TNFR2^+$ and $Ki\text{-}67^+$ cells (Fig 5~6). Thus, TNF-TNFR2 co-stimulation also activates $FoxP3^-$ Tconv cells in the tumor, although they have attenuated proliferative responses to TCR stimulation in vitro, which may be due to TCR signaling defects of Tconv cells in the tumor (37). Presumably the activated TIL Tconv cells become resistant to inhibition by LN-derived Treg cells from tumor free mice (Fig 8), but are still susceptible to inhibition by more highly activated tumor infiltrating Treg cells. Thus, TIL Treg cells which express higher levels of TNFR2 on a greater number of cells, may outcompete TIL Tconv cells in utilizing stimulatory factors, including co-stimulatory TNFR2 signaling, provided by the tumor environment. It was reported that both human and mouse Treg cells expressed high levels of surface TNFR2 and had the capacity to shed TNFR2 which contributes to the suppressive action of Treg cells (12). However, we did not detect elevated levels of soluble TNFR2 in either human or mouse $TNFR2^+$ Treg culture medium

under our in vitro experimental condition (data not shown). Furthermore, $TNFR2^{+}FoxP3/gfp^{-}$ T cells did not exhibit suppressive activity at all (Fig 2). Thus, our data suggest that shed TNFR2 may not represent a molecular basis for the immunosuppressive action of Treg cells. Instead, we hypothesize that Treg cells may outcompete Tconv cells in utilizing TNF as co-stimulatory molecules for cellular activation and expansion, which consequently further deprive Tconv cells of available TNFR2 ligand.

TIL Treg cells override the activation of Tconv cells and produce a dominant immunosuppressive environment in tumors. Further defining the mechanisms by which the tumor environment enhances the resistance of Tconv cells to Treg-mediated inhibition may be instrumental in devising effective immunotherapy to circumvent Treg activity and may yield anti-tumor immune responses. In contrast to tumor bearing states, the pathogenic activated effector T cells present in autoimmune conditions are more likely to outcompete Tregs in exploiting the TNF-TNFR2 co-stimulatory pathway. The possibility that this contributes to the therapeutic efficacy of anti TNF therapy in autoimmune diseases (38) merit future study.

Collectively, our study demonstrates that TNFR2 was predominantly expressed by activated Treg cells and to a markedly lesser extent expressed by activated Tconv cells, and enables both Treg cells and Tconv cells to respond to co-stimulatory signaling by TNFR2. Up-regulation of TNFR2 was much greater in tumor infiltrating Treg cells than Tconv cells and both subsets were activated to proliferate, presumably in response to both tumor associated antigens and stimulation by TNF enriched cytokine milleu in the tumor microenvironment. However, the activated intratumoral Treg cells still overcame the activated Tconv cells and resulted in the predominance of the immunosuppressive environment in the tumor. Selectively amplifying TNF-TNFR2 co-stimulation of Tconv cells and/or blocking its effect on Treg cells may be able to improve the outcome of immunotherapy and therefore merit future study.

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Chapter 8

Summary and concluding remarks

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Although TNF is a major proinflammatory cytokine, increasing evidence indicates that TNF also has immunosuppressive feedback effects. In the present thesis several questions were asked related to: the capacity of TNF to expand and to activate the function of T-regulatory cells, the receptor pathways required for these effects, and the ability of TNF/TNFR interaction to influence the stabilization of regulatory T-lymphocyte phenotypes.

A first answer to the question on the role of TNF for induction of Treg activity is presented in **Chapter 2** showing that, in both resting and activated states, mouse peripheral Tregs expressed remarkably higher surface levels of TNFR2 than Teffs. In co-cultures of Tregs and Teffs, inhibition of proliferation of Teffs by Tregs was initially transiently abrogated by exposure to TNF, but longer exposure to TNF restored suppressive effects. The profound anergy of Tregs in response to TCR stimulation was overcome by TNF, which expanded the Treg population. Furthermore, in synergy with IL-2, TNF expanded Tregs were even more suppressive. Thus, the stimulatory effect of TNF on Tregs resembles the reported costimulatory effects of TNF on Teffs, but is even more pronounced because of the higher expression of TNFR2 by Tregs (1).

TNFR2 is predominantly expressed by a subset of human and mouse Tregs. In **Chapter 3**, we characterized the phenotype and function of $TNFR2^+$ Tregs in normal and tumor-bearing C57BL/6 mice. We found that TNFR2 was expressed on 30-40% of the Tregs of the peripheral activated/memory subset that were most highly suppressive. In contrast, $TNFR2^-$ Tregs exhibited the phenotype of naive cells and only had minimal suppressive activity. Although not typically considered to be Tregs, $CD4^+CD25^-TNFR2^+$ cells nevertheless possessed moderate suppressive activity. In the Lewis lung carcinoma model, more highly suppressive $TNFR2^+$ Tregs accumulated intratumorally than in the periphery. Thus, TNFR2 identifies a unique subset of mouse Tregs with an activated/memory phenotype and maximal suppressive activity that may account for tumor-infiltrating lymphocyte-mediated immune evasion by tumors (2).

In **Chapter 4**, we report that human PB $FOXP3^+$ cells present in $CD25^{high}$, $CD25^{low}$ and even $CD25^-$ subsets of CD4 cells expressed high levels of TNFR2. Consequently, TNFR2-expressing $CD4^+CD25^+$ Tregs included all of the $FOXP3^+$ cells present in the $CD4^+CD25^{high}$ subset as well as a substantial proportion of the $FOXP3^+$ cells present in the $CD4^+CD25^{low}$ subset. Flow cytometric analysis of PB identified five-fold more Tregs, determined by FOXP3 expression, in the $CD4^+CD25^+TNFR2^+$ subset than in the

$CD4^+CD25^{high}$ subset. Furthermore, the $CD4^+CD25^+TNFR2^+$ subset expressed high levels of CTLA-4, CD45RO, CCR4 and low levels of CD45RA and CD127, a phenotype characteristic of Tregs. Upon TCR stimulation, human PB $CD4^+CD25^+TNFR2^+$ cells were anergic and markedly inhibited the proliferation and cytokine production of co-cultured T-responder cells. Therefore, the combination of CD25 and TNFR2 identifies a larger population of human Tregs, a population that may prove to be of diagnostic and therapeutic benefit in cancer and autoimmune diseases (3).

In **Chapter 5**, we show that TNF, in concert with IL-2, preferentially up-regulated mRNA and surface expression of TNFR2, 4-1BB and OX40 on Tregs. Agonistic antibodies against 4-1BB and OX40 also induced the proliferation of suppressive Tregs. Thus, TNF amplifies its stimulatory effect on Tregs by inducing TNF receptor superfamily (TNFRSF) members. In addition, administration of neutralizing anti-TNF Ab blocked LPS-induced expansion of splenic Tregs and up-regulation of TNFR2, OX40 and 4-1BB receptors on Tregs in vivo, indicating that the expansion of Tregs expressing these co-stimulatory TNFRSF members in response to LPS is mediated by TNF. Altogether, our novel data indicate that TNF preferentially up-regulates TNFR2 on Tregs, and this is amplified by the stimulation of 4-1BB and OX40, resulting in the optimal activation of Tregs and augmented attenuation of excessive inflammatory responses (4).

Several lines of evidence indicate the instability of $CD4^+FoxP3^+$ Tregs. An important question we posed at the beginning of the thesis was whether TNF also promotes the stability of Tregs, in addition to activate and expand of these cells. In **Chapter 6**, we reported that the proportion of Tregs in mouse strains deficient in TNFR2 or its ligands was reduced in the thymus and peripheral lymphoid tissues, suggesting a potential role of TNFR2 in promoting the sustained expression of FoxP3. We observed that upon in vitro activation with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab, FoxP3 expression by highly purified mouse Tregs was markedly down-regulated. Importantly, TNF partially abrogated this effect of TCR stimulation and stabilized FoxP3 expression. This effect of TNF was blocked by anti-TNFR2 Ab, but not by anti-TNFR1 Ab. Furthermore, TNF was not able to maintain FoxP3 expression by TNFR2-deficient Tregs. In mouse colitis model induced by transfer of naïve CD4 cells into $Rag1^{-/-}$ mice, the disease could be inhibited by co-transfer of WT Tregs, but not by co-transfer of TNFR2-deficient Tregs. Furthermore, in the lamina propria of the colitis model, the majority of WT Tregs maintained FoxP3 expression. In contrast, increased number of TNFR2-deficient Tregs lost FoxP3 expression.

Thus, our data clearly show that TNFR2 is critical for the phenotypic and functional stability of Treg in the inflammatory environment (5).

TNFR2 is also expressed by a small fraction of $CD4^+FoxP3^-$ Teffs in normal mouse, and its expression is up-regulated by T cell activation. This raises questions about the role of TNFR2 signaling in the function of Teff cells. In **Chapter 7**, by using FoxP3/gfp knock-in mice, we showed that TNFR2 signaling did not induce $FoxP3^-$ CD4 cells to become suppressive. Ki-67, a marker of proliferation, was concomitantly expressed with TNFR2 by CD4 cells, independent of Foxp3 expression, in normal mice and Lewis lung carcinoma-bearing mice. TNFR2 is associated with greater suppressive functions when expressed by Tregs and is associated with greater resistance to suppression when expressed by Teff cells. In mice bearing 4T1 breast tumor or Lewis lung carcinoma, intratumoral Teff cells expressing elevated levels of TNFR2 acquired the capacity to resist suppression by lymph node-derived Tregs. However, they remained susceptible to inhibition by more suppressive tumor-infiltrating Tregs, which expressed higher levels of TNFR2. Our data indicate that TNFR2 also co-stimulates Teff cells. However, intratumoral Tregs expressing more TNFR2 are able to overcome the greater resistance to suppression of intratumoral Teff cells, resulting in a dominant immunosuppressive tumor environment (6).

Concluding remarks

Both Teffs and Tregs are likely to use co-stimulation of TNF-TNFR2 for activation, expansion and maintenance of immune equilibrium. Based on the experimental evidence, we propose that, at the initial stage, TNF co-stimulates the activation of Teffs and liberates them from Tregs to mount an effective immune response against pathogens. At a later stage in the inflammatory responses, higher levels of TNFR2 expression by Tregs may enable them to outcompete Teffs for TNF and to down-regulate inflammatory responses. The stimulatory effect of TNF on Tregs thus represents an important negative feedback mechanism results in the attenuation and termination of prolonged or excessive immune responses, which otherwise may cause severe collateral damage (as reviewed in (7), see Figure 1).

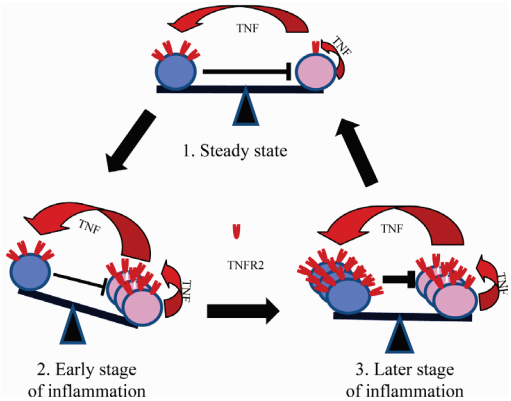

Figure 1: **Role of TNF-TNFR2 signal on Tregs and Teffs in the immune homeostasis and inflammation**. In the steady state, the equilibrium of the activation of Tregs and T-effector cells (Teffs) is crucial for immune homeostasis (1). In the early stage of inflammation, activated Teffs up-regulate their TNFR2 expression and increase their capacity to resist Treg-mediated inhibition, and therefore mount an effective immune response (2). In the later stage of inflammation, Tregs outcompete with Teffs for co-stimulatory TNF-TNFR2 action and enhanced their suppressive activity, resulting in the resolution of inflammatory responses and restoration of immune homeostasis (3).

In both steady and activating state, Tregs appear to express higher levels of TNFR2 than Teffs, indicating that TNF-TNFR2 costimulatory effect preferentially acts on Tregs. Although counterintuitive and contradictory to a previous report (8), our observation is supported by the emerging evidence from other investigators (9-15). For example, Mougiakakos and Kiessling et al found that TNF-TNFR2 interaction enhanced thioredoxin-1 expression on human Tregs, but not on Teffs, and therefore preferentially promoted Treg survival and activity within inflammatory milieu (10). Grinber-Bleyer and Salomon et al showed that pathogenic Teffs stimulated the activation of Tregs in vivo, at least partially through TNF-TNFR2 interaction (13). Housley and Clark et al have shown that TNFR2 is critical for the in vivo immunosuppressive function of naturally occurring Tregs (14). These studies substantiate the notion that TNF-TNFR2 interaction plays a critical role in the generation, expansion and function of human and mouse Tregs.

In the future, the clinical relevance of TNF-TNFR2 pathway in the activation of Tregs should also be addressed, which may improve the safety and efficacy of current anti-TNF treatment regimen. Furthermore, elucidation of signaling pathways and molecular basis of TNFR2 in the activation of Tregs may yield novel strategy to up- or down-regulate Treg activity for therapeutic purposes (16, 17).

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Samenvatting

Hoewel TNF als cytokine voornamelijk bekend is om zijn belangrijke pro-inflammatoire eigenschappen, duiden steeds meer aanwijzingen op een rol van TNF in immunosuppressieve 'feedback' mechanismen. Deze thesis beoogt meer inzicht te verwerven in de volgende vraagstellingen: (i) in hoeverre beïnvloedt TNF de functie en het fenotype van regulatoire T cellen (Tregs), (ii) welke receptoren zijn verantwoordelijk voor deze effecten en (iii) in welke mate beïnvloedt de TNF/TNFR interactie zowel effector T cellen (Teffs) als Tregs.

Onze verkregen resultaten tonen aan dat, in de muis, de expressie van TNFR2 op het celoppervlak van perifere Tregs beduidend hoger was dan in Teffs. TNF stimuleerde via TNFR2 voornamelijk de activatie en ontwikkeling van Tregs. De expressie van muis TNFR2 was kenmerkend voor een uniek subtype van Tregs, welke een geactiveerd geheugen fenotype hebben met maximale activiteit tot supressie.

In humaan perifeer bloed vertoonden de aanwezige $CD4^+$ cellen die zowel FoxP3-positief als mede ook $CD25^{high}$, $CD25^{low}$ en zelfs $CD25^-$ waren een verhoogde expressie van TNFR2. Dit wijst erop dat wanneer cellen die zowel CD25 en TNFR2 expressie vertonen een grotere populatie van humane Tregs identificeert dan wanneer enkel $CD25^{high}$ cellen in acht worden genomen. TNF veroorzaakte voornamelijk een verhoogde expressie van TNFR2 op Tregs. Dit expressieniveau werd versterkt door het stimuleren van 4-1BB en OX40, wat resulteerde in een optimale activatie van Tregs.

Meerdere studies benadrukken de instabiliteit van Tregs. Onze resultaten laten zien dat bij muizen die deficiënt zijn voor TNFR2 of voor liganden van TNFR2 het aantal Tregs in de thymus en in secundaire lymfoïde organen verminderd was, wat suggereert dat TNFR2 een mogelijke rol speelt in het bevorderen van FoxP3 expressie. TNF-TNFR2 interactie was cruciaal voor de fenotypische en functionele stabiliteit van Tregs, zowel in vitro als in vivo.

In muizen brengt een kleine fractie $CD4^+FoxP3^-$ Teffs ook TNFR2 tot expressie, wat gestimuleerd kan worden door T-cel activatie. Onze resultaten toonden aan dat enerzijds de specifieke expressie van TNFR2 door Tregs sterkere suppressieve functies impliceert en anderzijds expressie van TNFR2 door Teffs geassocieerd is met een verminderde suppressieve functie.

Zowel Teffs als Tregs gebruiken hoogstwaarschijnlijk de co-stimulatie van TNF-TNFR2 voor hun activatie, ontwikkeling en het behouden van een immunologisch evenwicht. Onze

bevindingen suggereren dat in de initiële fase tijdens ontsteking TNF de activatie van Teffs co-stimuleert en, los van Tregs, deze cellen in staat zijn om een efficiënte immuunrespons te genereren. In een later stadium van ontsteking kunnen Tregs door hun hogere niveau van TNFR2-expressie in competitie treden met Teffs voor TNF en hierdoor de inflammatoire respons inhiberen. Het stimulerende effect dat TNF uitoefent op Tregs representeert alsdusdanig een belangrijk negatief 'feedback' mechanisme dat uiteindelijk resulteert in een vermindering of het beëindigen van een langdurige of buitensporige immuunrespons.

In toekomstige studies is het derhalve niet onbelangrijk om de klinische relevantie van de TNF-TNFR2 interactie in de activatie van Tregs in kaart te brengen, wat kan resulteren in een verbeterde veiligheid en efficiëntie voor de huidige anti-TNF behandelingen. Het ophelderen van de betrokken signaalcascades van TNFR2 die leiden tot de activatie van Tregs zou mogelijk kunnen resulteren in nieuwe strategieën om de activiteit van Tregs te remmen of juist te verhogen.

Abbreviations

CLP: cecal ligation and puncture

cLP: colon lamina propria;

dLN: draining lymph note

FoxP3: forkhead box P3

KI: knock in

KO: knockout

LLC: Lewis lung carcinoma

LTα: lymphotoxin-alpha

LTβ: lymphotoxin-beta

MFI: mean fluorescence intensity

mLN: mesenteric lymph nodes

nCD4: naïve CD4 cells

non-dLN: non-draining lymph node

PB: peripheral blood

SP: single positive

Tconv cells: conventional T cells

Teff: $CD4^{+}Foxp3^{-}$ efector T cell

TIL: tumor infiltrating lymphocyte

TNFR2: tumor necrosis factor receptor type II

Treg: $CD4^{+}Foxp3^{+}$ regulatory T cell

Tregs: regulatory T cells

Triple KO: TNF/LTα/LTβ knockout

Curriculum Vitae

Xin Chen was born on February 14th, 1962, in Huangmei County, Hubei Province, China. He graduated from the Hubei University of Chinese Medicine, Wuhan, China with a Bachelor of Medicine degree in 1984, and obtained a Master of Medicine degree in 1987. In 1991, he earned the degree of Doctor of Medicine from the Guangzhou University of Chinese Medicine, Guangzhou, China. From 1991 to 1998, Xin Chen was a research physician at the Shenzhen Institute of Integrative Chinese and Western Medicine, Shenzhen Red Cross Hospital (Shenzhen 2nd People's Hospital). In 1998, Xin Chen started his Post Doctoral training at the School of Pharmacy and Biomedical Sciences, University of Portsmouth, UK. In April of 1999, Xin Chen joined the Laboratory of Molecular Immunoregulation, National Cancer Institute-Frederick, NIH, as a Visiting Fellow. In 2001, Xin Chen became a Scientist II in the Laboratory of Molecular Immunoregulation, Basic Science Program, SAIC-Frederick, Inc., NCI-Frederick. In 2011, he was promoted to Senior Scientist. Since 2000, he has published 43 papers in peer-reviewed journals, including 30 first-author papers, and 24 corresponding or senior-author papers.

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(* corresponding author)

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